

Seizing global warming as an opportunity

Preparations for the next global meeting on climate change, to be held in Kyoto in December, provide countries with an excuse to devise sound economic policies that make sense both scientifically and environmentally.

Britain's Labour party has always been slightly distrustful of the environmental movement. The reasons are not difficult to discern. One is that environmentalists tend to criticize the outcomes of those very processes — namely industrial and economic growth — on which the party's traditional supporters in the Labour movement rely as heavily for their welfare as do their employers. A second is that, historically, concern for the environment has many of its roots among landowning classes, keen to conserve it for their personal use. Red and green are colours that often do not sit well together.

US politicians and others therefore have cause for scepticism at the apparent conversion of the new Labour government to the environmentalist cause. Britain is planning to send an unprecedentedly high-powered delegation, headed by the prime minister, Tony Blair, to the United Nations meeting in New York later this month being held to mark the fifth anniversary of the Earth Summit, held in Rio de Janeiro in 1992. Officials say that Blair and his colleagues intend to take an aggressive approach, urging other countries to follow Britain's example in curbing greenhouse-gas emissions, for example, and chastising the United States for dragging its feet on policies to limit the hazards of man-made climate change (see page 640).

Those on the receiving end of these messages will no doubt be quick to point out that Britain's ability to set an example is as much the result of luck as of judgement. If, as expected, Britain becomes with Germany the only two major European states to meet a commitment made at Rio to reduce greenhouse-gas emissions to their 1990 levels by the end of the decade, this is primarily thanks to economic — not environmental — considerations that have prompted a shift from coal to gas as a source of energy. Indeed, it is ironic that Blair's ability to take the high ground is due partly to the success of the former prime minister, Margaret Thatcher, in crushing the power of a group that had been among Labour's strongest supporters, the coal miners.

Integrated approach

But justified scepticism must not be allowed to overshadow the main thrust of the new UK government's approach to climate change and other related environmental issues: that strategies designed to curb the unwanted side-effects of economic and industrial growth will only succeed if they are integrated into a broader patchwork of political and social goals. Domestically, Blair is seeking to do this by linking climate change policy to other priorities, sensible in themselves, such as energy conservation and a viable strategy for public transport. Internationally, a workable agreement on curbing greenhouse-gas emissions will only emerge if this takes into account the legitimate desire of developing countries for high levels of economic growth.

High among the countries learning its lesson the hard way is Japan. As host to the next meeting of signatories of the UN Framework Convention on Climate Change, due to be held in Kyoto in December, Japan will be expected to take a key role in devising a viable international strategy for implementing the emission cuts that are expected to be agreed at the meeting. But its own thinking on this issue appears hide-

bound by those government officials, particularly in the Ministry of International Trade and Industry, who appear locked into a commitment to the pursuit of economic growth at almost whatever cost (see page 641). A failure to shift this perspective will only weaken Japan's standing, both in Kyoto and in subsequent negotiations.

The ability of Blair and other politicians — including Japan's prime minister, Ryutaro Hashimoto — to set a new course depends heavily on good science. If agreement is reached, as hoped, at Kyoto on relatively ambitious targets for reducing greenhouse-gas emissions, much of the credit must go to the work of the Intergovernmental Panel on Climate Change (IPCC). The panel's hard-fought consensus, published in 1995, that "the balance of evidence suggests that there is a discernible human influence on global climate", has established a clear reality that cannot be wished away, as even lobby groups representing the fossil-fuel industries have now, reluctantly, come to admit.

Science still needed

And science's role is far from over. Much remains to be done in refining the models that are increasingly used as a guide to policy initiatives. One need is for credible models of regional impacts of global warming that are able adequately to characterize threats in terms of major perturbations in weather patterns, rather than single parameters such as a rise in temperature or sea levels. Inevitably, governments will be more tempted to take action if their voters are convinced of the self-interest in doing so; one of the biggest hurdles facing Clinton and his vice-president, Al Gore, will be to persuade the Senate to ratify any agreement reached in Kyoto, for precisely this reason.

But, even more than good science, also needed are imaginative solutions that promise both to achieve desirable environmental and social goals, and to be politically 'saleable'. Here Japan is already winning high marks in some quarters for a proposal for a two-tiered strategy to reduce greenhouse-gas emissions. While developed countries would be required to make immediate cuts, developing countries would be allowed to postpone any action until they have reached a previously agreed *per capita* level of carbon emissions. Although this would mean abandoning the 'flat-rate' reductions that many environmentalists claim is essential, others see in such a solution both an economic and a political logic that makes it attractive.

The greatest danger facing Blair and his colleagues, including Germany's chancellor Helmut Kohl, is that they will place an excessive confidence in the work of climatologists, using the apparent simplicity of broad conclusions to legitimate essentially non-scientific policies (as has happened with BSE). After all, some elements of the science will undoubtedly change — remember the 'limits to growth' debate — and policy must not be locked in concrete. Their greatest opportunity is that a growing sense of crisis over global warming will focus minds on the need to rethink our use of energy and other resources, and to accept the pain that this will inevitably require. If they can get this message across to a global audience, then both Clinton and Hashimoto will have reason to be grateful. □



'Quick' Mars mission proposal puts pressure on ESA's plans

[MUNICH] Proposals by the European Space Agency (ESA) to launch a relatively quick, cheap mission to Mars have sharply divided Europe's space science community over how to determine ESA's scientific priorities.

Last week, ESA's Space Programme Committee (SPC) approved the continuation of a feasibility study of the so-called Mars Express mission, which would analyse the surface of the planet, and might also collect surface samples (see *Nature* 387, 112; 1997).

But scientists working on the Planck Surveyor, a spacecraft that will survey the cosmic microwave background, fear that the Mars mission would either compromise the scientific content of or delay their own mission, because it competes for ESA's dwindling funds.

The Planck mission was selected last year as ESA's next medium-sized mission through the agency's rigorous system of peer-reviewed competition. In contrast, Mars Express has not undergone a formal selection procedure, but was proposed by ESA's science director, Roger Bonnet, for a variety of reasons, some of which are being seen as pragmatic rather than scientific.

One is that it could help to restore the frequency of missions in ESA's long-term Horizons 2000 science plan, whose scope has recently been reduced to meet budgetary pressures. It could also be done relatively cheaply, using spares of instruments destroyed when the Russian Mars 96 mission failed last year.

But Mars Express would have to be developed quickly, as the positioning of planets opens a route to Mars in 2003. And the agency has no spare cash, in particular following its decision to refly Cluster, the multisatellite mission that was lost when its Ariane-5 launcher exploded a year ago.

ESA's member states are already demanding changes in Horizons 2000 to allow the agency to launch small 'fast' missions — such as Mars Express which is politically attractive because of popular interest in the life-on-Mars issue — partly to keep data flowing to all parts of the space science community. These extra missions must be accommodated within ESA's fixed science budget, so the agency has been investigating ways of reducing the costs of all missions in the planning phase.

In particular, Bonnet has asked scientists and technical staff working on Planck and the 'cornerstone' Far Infrared Space Telescope (FIRST) to consider options for merging their missions, with a joint launch in 2005. Bonnet argues that a merged mission could save up to ECU400 million (US\$450 mil-

lion), nearly half of the planned costs of the individual missions. Although no formal link has been made, this would just cover the cost of the Cluster reflight and Mars Express.

Because they share some important characteristics, such as the need for deep cooling and an orbit far from Earth, the proposal to merge Planck and FIRST looked promising. But detailed studies now suggest that such a merger may be technically too difficult; for example, it seems unlikely that Planck instruments could directly share FIRST's liquid-helium cooling system.

Furthermore, even if the missions could be combined, scientists foresee arguments about which satellite should operate first; they cannot operate simultaneously, as Planck must conduct its year-long survey at a fast spin, which is incompatible with FIRST observations.

Studies of a possible merger are continuing. But, as a fall-back, the SPC has asked ESA to conduct a second study to determine whether the missions could be launched separately for the same level of saving without cutting back on the science, something Planck scientists fear may be impossible. Planck scientists fear they will come under pressure to reduce the scientific scope of the mission if they cannot make the savings, and that the mission may even be cancelled.

Lodewijk Woltjer, chairman of ESA's Space Science Advisory Committee, is an advocate of Mars Express. He points out that since last year's competition, two planetary missions — Mars 96 and Cluster — have been lost. He argues that the opportunity to make up for this at low cost justifies the fact that Mars Express was not reviewed in the normal way. "There was no time," he says.

But George Efstathiou, professor of astronomy at the University of Oxford and one of Planck's principal investigators, says that ESA should not have proposed a new project before plans for those already approved had been finalized. "Mars Express puts Planck in a very vulnerable position financially," he says. He also warns that ESA should not rely on potential financial savings on FIRST and Planck to expand Horizons 2000, "because we may not be able to do this".

Even if ESA did find money to cover its Horizons 2000 plans, astronomers would not have a clear path. While the ESA science budget provides launchers, satellites and technical support, participating member states are responsible for providing the sophisticated scientific instruments the satellites will carry.

At the SPC meeting last week, France, Germany, Italy and the United Kingdom expressed doubts about keeping up with the new-look Horizons 2000 plans. The issue will be discussed further at a special SPC meeting in September. This problem adds further uncertainty to the Planck and FIRST missions. FIRST was originally scheduled for launch in 2007, and member states might not be able to pay for the expensive FIRST instruments if a combined mission were brought forward by two years.

At last week's meeting, the SPC also approved a contribution of ECU15 million towards a new instrument for the Hubble Space Telescope as part of a scheme to ensure ESA's continuing involvement after 2001, when the current Memorandum of Understanding expires. Under the present arrangements, a minimum of 15 per cent viewing time is allocated to European scientists.

Alison Abbott

Move in sight for Greenwich observatory?



[LONDON] The Royal Greenwich Observatory (above), which moved to Cambridge in the 1980s, may be moving yet again — this time

to Edinburgh — if reported proposals from the Particle Physics and Astronomy Research Council are accepted (see page 646). □

ROYAL OBSERVATORY, EDINBURGH

Clinton sets a careful course on climate...

Signatories to the United Nations Framework Convention on Climate Change will meet later this year to decide on targets for greenhouse gas emissions. Our correspondents in Washington, London and Tokyo report on the prospects.

[WASHINGTON] President Bill Clinton is likely to promise "real action" to cut US greenhouse-gas emissions when he addresses a special session of the United Nations General Assembly in New York on 26 June, commemorating the fifth anniversary of the Rio treaty.

But despite anticipated pressure from the other government leaders at a G-8 summit in Denver, Colorado, before he goes to New York, Clinton is unlikely to specify at this stage what action he proposes. Instead, according to several government officials, the administration will wait until September to announce what emission targets the United States will support at the critical meeting of the parties to the Rio treaty in Kyoto, Japan, at the end of this year.

Senior administration officials met last week to discuss how Clinton should

approach concern about climate change that is expected to be raised at Denver by Tony Blair, the new British prime minister, and Helmut Kohl, the German chancellor. They also discussed the content of his address to the United Nations a few days later.

Debate continues to rage within the administration about how best to approach the issue. But optimism is growing among environmentalist groups that Clinton will share the view of Katie McGinty, his environmental adviser, and Jack Gibbons, his science adviser, that the United States must prepare for a Kyoto agreement that requires developed countries to take genuine steps to reduce greenhouse-gas emissions.

Observers strongly doubt that the US Senate will ratify such an agreement, however, or that the United States will itself do

anything to cut its emissions. These are now 7 per cent higher than they were in 1990, and projected to grow to 25 per cent above 1990 levels by the year 2010. Unlike Blair or Kohl, Clinton lacks the power to follow through directly on any agreement: he would have to persuade a deeply sceptical Congress that action is warranted.

But the administration may agree to cut emissions at Kyoto, and sell the idea to Congress later. "I'm more optimistic than I was three months ago that the administration will make real reductions in greenhouse-gas emissions," says Jonathan Lash, president of the World Resources Institute, a moderate environmental group, and co-chair of Clinton's Council of Advisors on Sustainable Development.

Clinton is likely to press other leaders at Denver for changes in their trade policies which will force trading partners in the developing world to plan for "sustainable development". He will also seek to impress them with demonstrations of 'clean cars' developed

Britain seeks leadership role with ambitious greenhouse-gas targets

[LONDON] Britain's new Labour government last week attempted to take the high ground in the climate change debate when it confirmed a pre-election promise to cut UK greenhouse-gas emissions to 80 per cent of 1990 levels by 2010, and announced a cabinet committee to coordinate environment policy across government.

But in a warning shot across the English Channel, government ministers said that the UK greenhouse-gas cuts would be conditional on commensurate reductions from Britain's 14 European Union partners, who have so far collectively agreed only to a 15 per cent reduction by 2010.

There were also harsh words from both John Prescott, the deputy prime minister who has taken on broad responsibilities for environmental issues, and Michael Meacher, the junior minister directly responsible for the environment, about the United States, which has refused to reveal its greenhouse-gas targets.

These announcements have raised the government's stock among environmentalists, who had been sceptical of the Labour party's apparent lack of

enthusiasm for environmental issues while in opposition. Greenhouse-gas cuts would come from imposing pollution taxes, promoting energy efficiency, greater use of renewable energy, and a new, 'integrated' transport policy that would put more emphasis on public transport.

Both ministers praised conservation bodies for highlighting the threat of global warming, despite being "dismissed as fringe groups". And they sharply criticized fossil-fuel industry lobbyists for "seeking to deny climate change, and undermining the science".

Speaking on World Environment Day last Thursday (5 June) in London, Prescott, secretary of state for a super-ministry combining environment, transport and the regions, broke with tradition when he praised his predecessor's attempts to raise the profile of environmental issues. John Gummer, secretary of state for the environment in the Conservative government, was popular among environmentalists for his robust stand on issues such as climate change.

But Prescott said the new government would "go further". His plans include a new parliamentary



Smoking gun? British ministers criticized fossil-fuel lobbyists for "undermining science".

environment committee, which would allow members of parliament to scrutinize government environment policy. And he revealed that Britain would field a five-strong team – including the prime minister, the foreign secretary and himself – at the United Nations special session in New York being held in two weeks to review progress since the 1992 Rio Earth Summit.

The previous day, Meacher, a former lecturer in social administration at the London School of Economics, had told a conference organized by the World Wide Fund for Nature (WWF) that the new government was "committed to leading the

fight against global warming in Europe and the world community". He added: "There is no question that climate change needs to be addressed urgently. It is already happening."

Meacher pointed out that it was chance, namely the economically driven decision to shut down most of the coal industry, rather than new policies, that had enabled Britain to achieve its targets on greenhouse gas emissions. He added that not enough had been achieved through "positive policies" such as improving energy efficiency in homes and transport. "As a result," he said, "carbon dioxide emissions will start to rise again in the first half of the next decade, and are projected to be back above 1990 levels by 2005 and go on rising afterwards."

Meacher said it was important for developed countries to set an example to the developing world, which is not yet committed to making greenhouse-gas reductions, but whose emissions are expected to outstrip those of developed economies within three decades. Meacher said that Britain expected Europe and the United States to show similar leadership.

Ehsan Masood

under the Programme for a Next Generation of Vehicles (PNGV), one of the US president's favourite technology programmes.

The administration is assessing the economic impact of any action the United States might take to cut emissions. But results of the analysis, which were due this month, are running late, and Everly Ehrlich, undersecretary of commerce and the official leading the assessment, has just left his post.

Nevertheless, the administration appears happy to announce its plans later rather than sooner, as this will minimize the opportunity for domestic criticism of them to build up before Kyoto. Any proposal for cuts is likely to come under sustained attack from opponents in US industry. Last week, the Business Roundtable, a powerful forum of 200 leading American business leaders, announced a \$1-million advertising campaign against such cuts in the run-up to the UN session.

And even if Clinton agrees to mandatory emissions cuts in Kyoto, the political outlook for substantive action on US greenhouse-gas emissions is uncertain, say observers on both sides of the debate. Such measures would be opposed not just by Republicans, who control both houses of the Congress, but by key Democrats as well.

John Dingell (Democrat, Michigan), for example, the senior Democrat on the Commerce Committee in the House of Representatives, is worried that they could hurt car manufacturers in Detroit, and Richard Gephardt (Democrat, Missouri), leader of the House Democrats, may oppose them to help win support among trade union leaders in preparation for his expected bid for the Democrat presidential nomination.

But the administration, prodded by the European states, may agree to emissions cuts in Kyoto and think about implementation afterwards. "Immediate ratification [of a Kyoto agreement] by the Senate is not the key," says Lash, who argues that a strong agreement there will put pressure on the Senate to take the threat of global warming seriously.

It would then be up to Clinton and to Al Gore, the vice-president and by far the strongest advocate of emission limits in the administration, to win treaty ratification in the Senate and support for enforcement legislation in both houses of Congress.

Gore, who has been keeping a low profile after some political embarrassments over fund-raising earlier this year, has recently been silent on the issue. Environmentalists argue that if he does not continue to take a firm stand on global warming, he will lose credibility in his own bid for the presidency.

But Gore's political handlers may view the matter differently. Like other US politicians, he has little to gain, but much to lose, by demanding economic sacrifice in response to a threat that does not affect the daily lives of most Americans. **Colin Macilwain**

...as Japan seeks to bridge split on emissions policy

[TOKYO] A lack of consensus between Japan's powerful Ministry of Trade and Industry (MITI) and its Environment Agency over the future reduction of greenhouse-gas emissions is delaying Japan's preparations for the United Nations (UN) climate change conference, to be held in Kyoto in the first week of December.

Signatories to the UN Framework Convention on Climate Change will be looking to Japan to guide the week's tortuous negotiations. But Japan's leadership is by no means guaranteed, as so far the host nation has yet to make up its own mind on legally binding limits to greenhouse gases from 2000.

While European countries, acting jointly through the European Union, have already proposed a 15 per cent reduction in emissions from 1990 levels (see *Nature* 386, 103; 1997), MITI and Japan's Environment Agency are having difficulty in agreeing on reduction methods and targets. According to officials in Tokyo, MITI is lobbying for a *per capita*-based proposal, whereas the Environment Agency wants a flat-rate reduction.

In an attempt to avoid mounting international embarrassment, Japan's foreign ministry last December brokered a compromise to allow Japan to put a broad proposal on the table. This consists of a flat-rate reduction in emissions for industrialized countries, and a *per capita*-based emissions ceiling for developing countries, whose emissions are still rising. This is being viewed by some as close to the ideal compromise during the Kyoto negotiations, where arguments will also centre on the question of emissions reductions for developing countries.

The United States wants to include developing countries in a broad protocol at some stage. But these countries will resist any flat-rate reduction as a threat to their industrialization plans. The idea of an emissions ceiling may be an attractive alternative.

But the two ministries are still divided on how, by how much and for how long Japan's own carbon dioxide emissions should be reduced from 2000. MITI appears to be basing its case on the argument that predictions of population growth imply an increase in demand for energy that will make a total reduction impossible.

Rather than revising its energy policy — already under severe political strain — MITI is therefore suggesting that *per capita* rates should be considered. Doubling the number of nuclear power stations in Japan might lead to a reduction in emissions, but it would take time, and recent scandals at the Monju and PNC reactors make this politically difficult.

As the host country for the Kyoto meeting, Japan is expected to lead the negotiations,



which involve about 150 countries. But the apparent discrepancy between Japan's international position and its internal disagreements has created concern among some foreign observers that this could reduce the chance of a successful outcome of what is likely to be a politically charged conference.

Some internal critics are also keen that, as host country, Japan should not sit on the fence and merely adapt its position to prevailing political forces at the last moment. But Japanese government officials say that they hope to finalize their position before the annual meeting of the Group of Eight (G8) major industrialized nations in Denver, Colorado, later this month.

Environmental topics — including the Kyoto meeting — are due to figure high on the summit's agenda, and Ryutaro Hashimoto, Japan's prime minister, is expected to propose that Japan hosts an international conference on freshwater supplies in developing countries later this year.

Nevertheless, some foreign observers in Tokyo feel that political pressure from overseas will be needed to encourage Japan to make the compromises it needs to decide on carbon dioxide emissions. Some feel it is a decision that may, unusually, have to be taken at cabinet level.

Japan is expected to be severely criticized at Kyoto by nongovernmental organizations for its lack of success in reducing emissions so far. At the 1992 meeting in Rio, Japan and other industrialized nations promised to reduce emission to 1990 levels by 2000, but on current projections Japan is unlikely to meet this target (see *Nature* 387, 447; 1997).

In 1995, Japan's carbon dioxide emissions increased by 0.5 per cent to a record 332 million tonnes, an increase of 8.3 per cent on 1990. Many other countries, including the United States, are also unlikely to meet these targets. But a particularly awkward spotlight is expected to fall on the host country's record at the conference.

Richard Nathan

Arecibo enters 'new era' in astronomy

[BOSTON] After extensive renovations, jointly funded by the National Science Foundation and the National Aeronautics and Space Administration, an inauguration ceremony will be held on Saturday (14 June) at the Arecibo Observatory in Puerto Rico, the world's largest radio telescope.

The \$26-million project has taken five years to complete — twice as long as expected. However, scientists are delighted with the overall result, which will greatly increase the telescope's performance and lead to what is described as a 'new era' in radioastronomy.

"These are the most dramatic changes made to any radio telescope, and perhaps to any existing telescope," says Donald Campbell, associate director of the National Astronomy and Ionosphere Center (NAIC), which is based at Cornell University and which operates Arecibo. He points out that when Arecibo opened in 1963, it was generally assumed that it would operate for only about 10 years. But the "soundness of the original design" has allowed major improvements to be made over the decades.

The latest upgrade involved three main tasks. First, a 16-metre-high screen has been built around the perimeter of the telescope to reduce thermal radiation from the ground and block out extraneous radio noise.

Second, a 'gregorian' subreflector system, using two mirrors housed in a 90-tonne dome suspended high above the main dish, has been erected to send signals to the planets

and asteroids, picking up the return scatter to analyse for rotation, surface features and temperature. It is also used extensively to investigate the Earth's upper atmosphere.

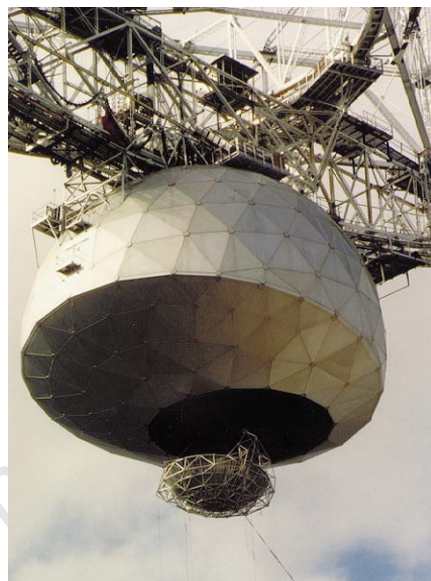
Finally, a more powerful radar transmitter has been installed. The receivers can now collect more of the photons that fall on the primary dish.

Most of the construction work was completed last autumn, and astronomers are now fine-tuning their revamped instrument, planning to resume observations later this year. Says Campbell: "It takes a while to get a new telescope fully operational, and we have to think of this as a new telescope, since just about everything has changed, save for the primary reflector."

The increased versatility promises exciting results. According to Paul Goldsmith, director of NAIC: "The range of frequencies we can look at has roughly expanded by a factor of five, while the instantaneous bandwidth — which means the chunk of the spectrum we can examine at any one time — has increased 20-fold".

Furthermore, the greater sensitivity of the new gregorian system will permit astronomers to study weak sources that could not be seen before. The improved performance will boost the hunt for new pulsars and pulsar planets, as well as the investigation of star-forming regions.

But there will also be substantial gains for those studying objects in the Solar System.



Eye in the sky: The sub-reflector system is raised into the radome of Arecibo radiotelescope.

"We had the world's most powerful radar telescope before, and we've improved it by a factor of 20," says Campbell. "That will enable us to survey about 10 times as many asteroids and comets with very high resolution."

One peripheral activity to benefit from Arecibo's enhanced sensitivity and wider frequency coverage is the search for extraterrestrial intelligence (SETI). SERENDIP, a SETI project based at the University of California, Berkeley, will begin 'eavesdropping' this year with a new and improved receiver.

According to Woody Sullivan, an astronomer at the University of Washington in Seattle, one project, called SETI@home, intends to analyse chunks of SERENDIP data in greater detail, producing what would be the equivalent of a whole new survey. Provided that funding can be secured, Sullivan hopes to line up 50,000 or more volunteers, who would each be given software to process the data on their home or office computers while operating in screensaver mode. This would eliminate the need for — and cost of — a supercomputer. "If we could keep that up for two years, we should have a scientifically significant survey," he says.

Meanwhile, astronomers at Cornell are planning the next round of improvements at Arecibo. One task will be to reset the 38,778 aluminium panels that form the giant antenna, to improve its accuracy and permit detection of shorter-wavelength signals.

The panel-setting project, estimated to cost \$1 million, could begin within a few years. "We have an obligation to keep this instrument viable," says Mike Davis, project scientist for the upgrade project. "When you have the world's biggest telescope, you have a responsibility to push the system to the limits."

Steve Nadis

'No change' while NIH revises peer review

[WASHINGTON] Procedures for grant applications to the US National Institutes of Health (NIH) will remain unchanged for now, even though the agency is in the process of overhauling its peer-review system.

According to Wendy Baldwin, deputy director for extramural research at NIH, which distributes more than \$5 billion a year in grants, there will be no substantial changes to the application forms used by external scientists for up to two years, as it is planned to combine them with the launch of a new system of electronic applications.

Until then, said Baldwin, "the astute applicant is going to be more up front about these features in what they write". This is a reference to five new criteria for judging grant proposals, announced by Harold Varmus, the NIH director, last month.

The five criteria are: significance, approach, innovation, investigator and environment. For now, they will simply be outlined in the instructions to applicants that accompany application forms. In these, "it will be clear" to applicants what the criteria are, Varmus said last week.

One of the five criteria has proved controversial: the demand for "innovation". As outlined by Varmus last month, peer-reviewers will ask of an application: "Does the project employ novel concepts, approaches or methods? Are the aims original and innovative? Does the project challenge existing paradigms or develop new methodologies or technologies?"

Some scientists complain that the "innovation" criterion will work unfairly against clinical researchers. But others back it strongly, most prominently among these Keith Yamamoto, a molecular biologist at the University of California, San Francisco, who chairs the panel that advises the NIH's Division of Research Grants.

The NIH is also overhauling its system of peer review, with changes likely in structure, membership and subject areas of study sections. The effort is being led by Elvera Ehrenfeld, a molecular biologist from the University of California, Irvine, who is the new head of the Division of Research Grants. She expects to propose a reorganization within 18 months.

Meredith Wadman

Germany follows science council's advice on closures

[MUNICH] For the first time since reunification, German federal and state governments have accepted without a fight a recommendation of Germany's science council, the Wissenschaftsrat, to close two 'blue-list' research institutes.

Last week, the Bund-Länder Kommission (BLK), which coordinates national and regional science policy, decided to withdraw funding from the Institute for Mineral Oil Research, in Lower Saxony, and the Institute for Child Nutrition, in Dortmund, Nordrhein-Westfalen. The Wissenschaftsrat had judged the quality of research in the two institutes to be low.

The BLK's decision is a boost to the status of the council, an independent government advisory body made up of leading scientists and political representatives, whose advice has often been ignored. Blue-list institutes, which are jointly funded by *Länder* and federal governments, have proved hard to close, partly because of the social costs of doing so.

Since reunification the number of such institutes in Germany has increased dramatically, from 48 to 82 (see *Nature* 379, 669; 1996). Three years ago, the BLK asked the Wissenschaftsrat to review the scientific quality and administrative efficiency of the institutes. But although the Wissenschaftsrat has so far recommended that funding be withdrawn from 7 of 21 institutes assessed, host *Länder* have, until now, always successfully contested these conclusions.

The BLK's apparent reluctance to take firm action, despite a statement last year from the federal research minister Jürgen Rüttgers that he wanted all Wissenschaftsrat recommendations to be implemented, had caused some council members to question the value of their efforts. "If politicians ask for our advice, we expect it to be heeded," says Michael Maurer, a spokesman for the Wissenschaftsrat, adding that last week's decision "is certainly what we wanted".

Two factors helped the BLK to agree on the closures. The first is Germany's worsening budgetary problem, and the second a recent agreement that redundancy payments should be jointly financed by federal and *Länder* governments, rather than by the host *Länder* alone. "This makes it less difficult to close blue-list institutes," says BLK member Eberhard Wagner.

But the BLK still wavers. Two further institutes recommended for closure received a temporary reprieve last week. An Earth sciences institute in Hannover and an environmental health institute in Düsseldorf have both been asked to present new scientific strategies.

Quirin Schiermeier

Japanese budget austerity puts science plans at risk

[TOKYO] A stringent fiscal reform package announced last week by a government panel headed by the prime minister, Ryutaro Hashimoto, has thrown into doubt Japan's ambitious plan to increase public-sector funding for science and technology by more than 50 per cent over the next five years.

The fiscal reform plans approved by the cabinet call for severe cuts in government spending to rein in the ballooning national debt which stands at more than ¥400,000 billion (US\$3,450 billion). Most of the cuts will be directed at public works spending and overseas development assistance, but science and technology will not escape unscathed.

In June last year, the government approved a plan to increase public-sector funding for science and technology by more than 50 per cent by 2001 (see *Nature* 381, 725; 1996). This fiscal year's budget, which came into effect on 1 April, accordingly has large increases for all science-related ministries and agencies, in many cases exceeding 10 per cent (see *Nature* 385, 104; 1997).

But after last week's reform proposal, outlays for next fiscal year are likely to be held to a 5 per cent increase, and increases in subsequent years are likely to be even smaller. According to an official at the ministry of finance, increases may remain low until at least 2003, when the government hopes to achieve its goal of reducing the combined deficits of central and local government to

3 per cent or less of gross domestic product.

The reform plan also states that no new large-scale science projects will be approved during this period of restraint, and existing large-scale projects that are experiencing trouble — such as the Monju fast breeder reactor — will be reviewed and either reduced in scale or terminated.

Furthermore, the panel echoes recent calls for strict external evaluation of public research bodies and projects. In particular, government-funded national research institutes, which constitute only a relatively small portion of the public-sector research system in terms of numbers of researchers, but which consume a large share of the overall research and development budget, are targeted for evaluation and reform.

Such institutes have benefited financially from a series of recent supplementary budgets. But critics argue that money has simply been poured into the purchase of expensive equipment, with little long-term planning or strengthening of research personnel.

The budget recommendations reflect a broader feeling that Japan may not be able to meet its promise to maintain the rapid expansion of its science base. In a recent article, the *Nihon Keizai Shimbun*, Japan's influential business newspaper, stirred a debate with an article describing what it called the 'research money bubble', suggesting that it may soon burst as did the investment 'bubble' of the 1980s.

Robert Triendl

Spending cuts threaten ambitions to host ITER

[TOKYO] Japan's ambitions to host the International Thermonuclear Experimental Reactor (ITER) may fall victim to the new calls for restraint in government spending (see above).

Last year, ITER's other partners, the United States, the European Union and Russia, hoped that Japan might come to the rescue of the faltering international project when Japanese officials hinted that Japan might shoulder 70 per cent of construction costs if the reactor is built in Japan (see *Nature* 380, 655; 1996).

But the fiscal reform package announced by the Japanese government last week specifically states that

ITER will not be invited to Japan during the next three years of restraint.

Furthermore, an official from the ministry of finance says that funding for such large-scale science projects will remain severely restrained until at least 2003 and probably beyond.

Despite the constraints, officials of the Science and Technology Agency, which funds Japan's participation in the present engineering-design phase of ITER, still hope to host the reactor. "We still want the ITER project to be realized, if possible with Japan as one of the potential hosts," says Satoshi Tanaka, director of the agency's office of fusion energy.

Furthermore, he points out that, in any case, ITER partners are likely to agree to a three-year 'transitory' phase between the end of the engineering-design phase next year and the start of construction because of the financial difficulties that all ITER partners are facing.

The Federation of Economic Organizations (Keidanren), a powerful industry association, also continues to promote the idea of constructing ITER in Japan. A Keidanren spokesman claims that, as a result of Keidanren solicitations, Korean and Taiwanese government agencies are "very keen" on the idea. **Richard Nathan & R. T.**

White House bill would ban human cloning

[WASHINGTON] Acting on the advice of his bioethics advisory commission, President Bill Clinton on Monday sent to Capitol Hill legislation banning anyone in the public or private sector from using cloning to create children. The legislation would expire in five years, forcing a review at that time.

Clinton said he was acting on a "national consensus" that "attempting to clone a human being is unacceptably dangerous to the child and morally unacceptable to our society". Clinton said that the bill will not ban animal research or the cloning of DNA in cells, and argued that the cloning of a sheep reported by Scottish scientists in February promises "revolutionary new medical technologies".

But his bill, he said, "will ensure that we do not fall prey to the temptation to replicate ourselves at the expense of those beliefs and the lives of innocent children we would produce". Clinton also said that his March ban on federal funding for cloning human beings will remain in place "until the day I sign the legislation into law". And he reiterated his call to the private sector to refrain from attempting to clone humans.

Under the Clinton bill, the National Bioethics Advisory Commission (NBAC), would be required to continue studying cloning, reporting to Clinton again six months before the law expired.

The NBAC last week decided it was premature to reach a position on whether the cloning of humans to produce offspring is ethically acceptable or not, calling only for a provisional legislative ban on the grounds of safety.

In a report submitted to Clinton on

Monday, the NBAC said that the need for a permanent law remained "an open question" for Americans to decide, given the "more speculative" nature of ethical questions concerning the possible psychological risk to clones, and cloning's impact on social mores. These, it wrote, "may or may not be enough to justify prohibitions in the future".

Although the commission described human cloning for reproductive purposes as "morally unacceptable" at present, it based its recommendation on safety considerations. It argued that "the use of this technique to create a child would be a premature experiment that would expose the fetus and the developing child to unacceptable risks".

Indeed, Dolly, the first animal to be produced using nuclear transfer cloning techniques, was the only cloned lamb to result from 277 fusions involving adult cells, and no information is yet available on her longevity, fecundity or susceptibility to diseases such as cancer.

In its report, the commission said that safety concerns alone were "sufficient to justify a prohibition on cloning human beings at this time". At the same time, it acknowledged that the use of such techniques might be "characterized as the exercise of a fundamental [constitutional] right to attempt to procreate". In a further indication of the uncertainties in the debate, the commission recommended that any legislation should automatically expire after three to five years, in order to force a review of its appropriateness as science evolves.

In a move that drew praise from some scientists, NABC did not call for restrictions

on cloning research that stops short of implantation, leaving open the possibility that researchers in the private sector could use the technique to create embryos for *in vitro* studies. This is "a good thing", said Roger Pedersen, a mammalian embryologist at the University of California, San Francisco. "This kind of research could have tremendous value to people."

While not the intent of the 18-member commission, this exception might in practice open the way to resolving some safety concerns of human cloning, and bring a step closer the feasibility of cloning human offspring. Although federal funding for human embryo research is currently prohibited by law, such research is allowed in the private sector.

In recommending the broader law against cloning for implantation, the commission argued that "the history of infertility treatment — especially that of *in vitro* fertilization — demonstrates that where there is a sizeable and well financed demand for a novel service, there will be professionals willing to try to provide it".

NBAC cautioned that legislation should be carefully written "so as not to interfere with other important areas of scientific research" such as cloning of cell lines and DNA sequences.

But some scientists regret the call for legislation. "This would add strength to the effort to criminalize other aspects of [cloning] research, including perhaps *in vitro* studies," says Pedersen. "It provides an avenue for politically based regulation of research."

Meredith Wadman

US opens reagent repository to boost malaria vaccine research

[WASHINGTON] A reagent repository intended to give a 'jump start' to malaria vaccine research by supporting established investigators and attracting new scientists into the field is being opened by the US National Institute of Allergy and Infectious Diseases (NIAID).

"Everything from insects through malaria antigens [to] immune sera [will be] made freely available to any investigators who have a good faith effort in wanting to do malaria research," Anthony Fauci, the director of NIAID, said last week.

Fauci announced the plan for the repository — part of a ten-year NIAID malaria vaccine development strategy — at a meeting of the Advisory Committee to Harold Varmus, the director of the National Institutes of Health (NIH).

The proposal grew in part from an international meeting on malaria research held in Dakar, Senegal, in January, in which

Fauci and Varmus participated (see *Nature* 386, 539; 1997). Both will travel to a follow-up meeting in the Hague early next month at which participating organizations hope to agree on a collaborative international system to review and fund malaria work.

According to Varmus, the meeting at the Hague will provide a "pivotal moment" in which participating groups will decide whether to adopt "a radical approach of putting money in a common pot", an idea he raised in Dakar.

Alternatively, the groups could in principle decide to stick with the status quo. Or they may decide to develop a common application and review process, but allow individual organizations to maintain control of their own money by choosing which of the projects approved by the common review body they will fund.

While NIH money will be at stake, Varmus said, "also at stake will be the

fortunes of the people in the world who are affected by this disease". Fauci said that the amount of money made available under any of these schemes will depend on the quality of the research ideas solicited since January. At the high end, he said, the figure might reach \$50 or \$100 million.

At last week's NIH meeting, Fauci said he plans to use between \$200,000 and \$400,000 "immediately" to start a repository housed initially within existing NIAID contract operations near NIH's campus in Bethesda, Maryland, but soon supported by its own \$1–\$1.5 million yearly contract. The money would augment the NIAID's current malaria budget, estimated at \$21.8 million in 1997.

The goal, according to Fauci, is "to allow malaria investigators to compete effectively against the broad pool of [grant applicants]". In the malaria vaccine research field, "you can't do that unless you have some of these reagents".

M. W.

French science set to shift up a gear

Declan Butler

France's new socialist prime minister, Lionel Jospin, made a promising start last week to fulfilling a pledge to make research a national priority.

The creation of an independent ministry for national education, research and technology has given a prominent political status to research in Paris. Responsibility in the previous administration was relegated to a junior minister; in contrast, the new ministry ranks third in importance after the ministries of employment and of justice in the ministerial hierarchy.

The appointment of well-known geologist Claude Allègre as minister for education, research and technology has also been broadly welcomed by scientists (see below). His reputation for single-mindedness is widely judged as precisely what is needed to overcome the huge problems facing French universities and research organizations, including widespread resistance to reform.

The new government is not expected to outline its policies until next week. But during the election campaign the Socialist party promised to reverse the cutbacks in research budgets and jobs made since the conservatives came to power in 1993.

Allègre's hand should be strengthened in talks with the finance ministry by the fact that he is one of Jospin's closest friends and advisers, and by the political weight of his secretary of state for education, Ségolène Royal.

The government's promises have also pleased trade unions. They have demanded recruitment of 5 per cent annually — or 550 researchers — over five years to offset the threat to a sensible age structure in laboratories posed by the expected wave of retirement between now and 2005, when more than half of the French research workforce retires.

Indeed, the victory of the Left has been

broadly welcomed by scientists who remain deeply distrustful of the Right's commitment to research. Part of this distrust stems from the 1986 government's attempt to dismantle the Centre National de la Recherche Scientifique (CNRS), and to impose hasty reforms of the university system.

Later conservative governments recognized that imposition could lead to unpopularity. But they failed to produce workable policies, leading one critic to remark: "There has been no pilot in the plane."

Six months ago, for example, Guy Aubert, the director-general of the CNRS, was poised to introduce a broad reform of his organization. But François d'Aubert, then secretary of state for research, told him to put it in a drawer until after the general elections — at the time scheduled for next year.

Observers in Paris believe the main impact of the Left's victory may be to end this period of policy paralysis. They argue that substantial increases in support for science are unlikely given the pressure on funds. However, a reversal of the downward trend should ease tensions between the government and the research community, thus engineering a climate more conducive to reform.

Optimism is also being generated by the personal credentials of both Jospin and Allègre. Jospin has a genuine enthusiasm for education and research, and his approach as minister of education from 1988 to 1992 is widely considered as having been both energetic and progressive. "It's the best government [for science], at least on paper, that we have had for a long time," says Pierre Chambon, head of the Institute of Genetics



Entente cordiale: President Jacques Chirac with education minister Claude Allègre (right).

and Molecular Biology near Strasbourg.

Allègre himself was special adviser to Jospin when the latter was minister of education. Together, they launched an ambitious expansion plan for the universities — and introduced measures to give universities much greater autonomy. In the recent election campaign, Allègre promised that the Left would accelerate both actions and embark on a complete rethink of the research and education system.

One of the major problems that the new government will face stems from the status of civil servant enjoyed by all public researchers. Salaries often account for more than three-quarters of the running costs of research agencies, leaving individual scientists short of the resources needed for meaningful research, and denying the agencies the means to develop strategic programmes.

But any idea of abandoning the system of universal life tenure in favour of the competitive short-term contracts common in Anglo-Saxon countries remains taboo in France. Given that there is little prospect of massive increases in the research budget, the new government will be obliged to make better use of existing resources, says Chambon, arguing that this in turn will mean tough reforms to reduce the share of research organizations' budgets taken up by salaries.

Some worry that Allègre's impatience for reform is a recipe for conflict between the government and the scientific community — he is a controversial personality, known for his autocratic tendencies and a talent for making enemies. But many seem willing to give him the benefit of the doubt, arguing also that Allègre will ultimately have to modify his approach in line with the government's commitment to negotiated reform.

Says Chambon, "I prefer someone who makes the occasional mistakes to someone who doesn't move; because we haven't moved for years now; nothing is being done and we are falling into mediocrity. Allègre is the only person who can shift the universities and research; he has original ideas and vision, and if he can't succeed then no-one can." □

Geologist takes over the ministerial helm

[PARIS] Claude Allègre, France's new minister for national education, research and technology, holds the 1986 Crafoord Prize, awarded annually by the Royal Swedish Academy of Sciences for outstanding basic research in disciplines not covered by the Nobel prizes.

A geologist, Allègre has held the chair of life science at the University of Paris-VI,

headed the Institut de Physique de Globe in Paris from 1976 to 1986, and was president of the French geological survey — the Bureau des Recherches Géologiques et Minières — from 1992 until last month, when he was fired by the outgoing government.

Allègre is no stranger to politics. He became a member of the steering

committee of the Socialist party in 1987, and a member of its executive board in 1990, resigning in 1992 in protest at what he described as the party's lack of ideas.

Allègre himself was a member of the European Parliament from 1989 to 1994, and has been *conseiller régional* (regional councillor) of the Languedoc-Roussillon region since 1992.

Observatory merger plans prompt protest by Astronomer Royal

[LONDON] Tensions are running high in parts of the British astronomy community over reports that the Particle Physics and Astronomy Research Council (PPARC) has recommended that the Royal Greenwich Observatory (RGO), currently based in Cambridge, be merged with the Royal Edinburgh Observatory, and its activities moved to the latter's Edinburgh site.

Sir Martin Rees, who is both Royal Society professor of research at the University of Cambridge and the Astronomer Royal, is due to meet the science minister, John Battle, this week to protest at the way the proposal has been reached, and at the disruption that combining the two organizations, which provide technical support for British telescopes in the Canary Islands and Hawaii, is likely to cause.

It is less than ten years since the RGO moved to Cambridge at a cost of £6 million (\$9.6 million) from its previous site at Herstmonceux Castle in Sussex, where it had been located since moving from its original site in London. PPARC officials, however, are believed to have told the government that the amalgamation of the observatories represents the best use of funds available for astronomy research, and many astronomers are keen that uncertainty over the observatories' future is resolved.

'US deal means huge cut in science spending'

[WASHINGTON] The budget deal reached last month by President Bill Clinton and the US Congress would cut expenditure on science and technology by 16 per cent in real terms by 2002, according to an assessment by George Brown (Democrat, California), the senior minority member on the Science Committee in the House of Representatives.

"Neither the president nor Republican congressional leaders acted to protect research and development programmes in the budget agreement," says the 77-year-old Brown, who has announced plans to run again next year in his San Bernardino district for a fourteenth term. The non-binding agreement includes new and steeper cuts in the account which funds the National Aeronautics and Space Administration, the National Science Foundation and basic research at the Department of Energy.

Varmus panel to study patent constraints

[WASHINGTON] Harold Varmus, the director of the US National Institutes of Health (NIH), has set up an expert panel to explore

ways of easing restrictions on access to proprietary materials and methods by research scientists.

Varmus announced last week that he has asked an *ad hoc* subcommittee of his director's advisory committee to report to the full committee on the issue in December. The subcommittee will be led by Rebecca Eisenberg, a law professor and intellectual property expert at the University of Michigan, and will include John Barton, a professor at Stanford Law School.

Varmus said that his action was prompted by "a lot of anxiety in the research community" about access to materials ranging from genetic tools to engineered mice. He said that "undue restraints" on scientists' access may be adversely affecting public health, for example when licences on patented materials impose extensive restrictions on their downstream dissemination and use.

French minister plans to close Superphénix

[PARIS] Dominique Voynet, France's new environment minister, has promised to close down Superphénix, the country's troubled 1250-MW fast breeder reactor, which was designed as a prototype commercial power plant and later reincarnated as a research reactor (see *Nature* 385, 104; 1997). Voynet is one of the eight members of the French green party, Les Verts, who were elected to the National Assembly in this month's general election, the first time the party has obtained seats in the assembly.

Shutting down the reactor will require the creation of a scientific committee to oversee the operation, and Voynet will also need to submit to the government a plan explaining what she intends to do with its staff and how to compensate both the local communities and the German and Italian utilities that are France's partners in the project.

Georgia Tech sees off website challenge

[LONDON] The French campus of the Georgia Institute of Technology has won the right to retain English as the sole medium of its World Wide Web site. Two French-language watchdog organizations had sued the campus of Georgia Tech in Lorraine for allegedly breaching a law that foreign language advertising must be accompanied by a French translation.

The case was thrown out on technical grounds; the 1994 French law requires a police investigation of a potential breach to be conducted as a precondition to any civil action, and both organizations had failed to notify the police. The university, however, has now decided to provide information in German and French. A spokesman for

Georgia Tech had argued that the website was in English because that is the sole medium of instruction at the university.

Senators pull back from NIH funding initiative

[WASHINGTON] Although they voted in support of a resolution approved unanimously by the US Senate last month to double funding for the National Institutes of Health (NIH) over the next five years, many senators now appear reluctant to urge a key colleague to allocate funds to that end.

The symbolic "sense of the Senate" resolution was authored by Connie Mack (Republican, Florida) and Diane Feinstein (Democrat, California). All 98 senators present in the Senate on 21 May voted in favour of the resolution. Now, Mack and Feinstein have asked all of their colleagues to sign a letter to senator Ted Stevens (Republican, Alaska), the chairman of the Senate Appropriations Committee.

The letter urges him to begin doubling the NIH's \$12.7 billion budget by giving it \$2 billion in new money in the 1998 funding bill, just as the resolution of 21 May demanded. But so far, 44 of the 98 senators who voted for the resolution have declined to sign the letter addressed to Stevens.

China aims to boost science funding

[LONDON] China's vice-minister in the State Science and Technology Commission, Deng Nan, has promised to increase next year's scientific development budget by 14.2 per cent. Banks will also offer nearly 20 billion yuan (US\$2.5 billion) in loans for scientific research.

Speaking at the closing session of a national conference on conditions in science, Nan said China will focus its extra support for basic research, and the setting up of state laboratory animal breeding centres, data banks for plant seeds, and information networks. Nan also confirmed that, by 2000, China will spend 2 billion yuan setting up a number of scientific installations.

From UK research board to Lords committee

[LONDON] Lord (David) Phillips of Ellesmere, the last chairman of Britain's Advisory Board for the Research Councils, is to become the new chairman of the House of Lords Select Committee on Science and Technology. Phillips, who was a professor of molecular biophysics at the University of Oxford between 1966 and 1990, takes over from Lord Selborne, chancellor of the University of Southampton and a former chairman of the Agriculture and Food Research Council.

Funding for malaria genome sequencing

Sir— We should like to correct a statement in your Briefing on malaria about funding for the sequencing of the genome of the malaria parasite *Plasmodium falciparum* (*Nature* **386**, 535–540; 1997).

You report that the US Department of Defense, the US Burroughs Wellcome Fund, the UK Wellcome Trust and the US National Institutes of Health (NIH) have created a fund for this sequencing effort. In fact, no 'fund' has been established and there has been no pooling of funds among the donors. However, an exciting international consortium of scientists and funding agencies has been formed during the past year to provide the funds and reagents required to sequence the *P. falciparum* genome, and to produce, annotate and publish these sequences.

We estimate that it will cost a minimum of \$15 million to complete and annotate the 30-megabase *P. falciparum* genome. In the initial pilot phase, funding has been used to develop methods and to produce the reagents required for the high-throughput sequencing and for annotating the sequences. As a result of progress so far, the plan is to sequence the 14 chromosomes separately and to divide the work by chromosome, with about half the work being done in the United States and half in England. The work in the United States has initially been supported by grants from the NIH and the Burroughs Wellcome Fund and in England by the Wellcome Trust. Subsequent support will also be provided

by the US Department of Defense. In the United States, the Naval Medical Research Institute is providing *P. falciparum* DNA and chromosomes, the Institute for Genomic Research will be doing a significant amount of the high-throughput sequencing and Stanford University is committed to sequencing at least one of the larger chromosomes.

The donors are also supporting research on clone stability, library construction and optimization of sequencing reagents for the project at these three institutions, and at Roswell Park Cancer Institute and Harvard University. In England, the Institute of Molecular Medicine at the University of Oxford is providing *P. falciparum* reagents, and sequencing is being done at the Sanger Centre at Cambridge. It is expected that during the next few years a number of other laboratories may participate in the effort.

The participants (scientists and donors) involved in this collaborative undertaking coordinate their activities through conference calls, e-mails and meetings. The researchers and funders met in Baltimore, Maryland, in December 1996 and will meet in Cambridge, England, on 16–17 June 1997. In addition, there has been an effort to involve the broader malaria community in this project and to facilitate widespread dissemination of the genomic information.

There are substantial scientific obstacles to be overcome before the sequence of the *P. falciparum* genome is

fully elucidated. Nonetheless, during the past year we have made great strides in identifying the funds required to pursue the project, building a strong group of scientists and institutions to execute the science, and establishing a collaborative network of scientists and donors. We believe that this project will provide a road map for malaria research in the twenty-first century, research that will lead to improved treatment and prevention of a parasitic infection that causes hundreds of millions of illnesses, and millions of deaths annually.

Stephen L. Hoffman

*Malaria Program,
Naval Medical Research Institute,
Bethesda,
Maryland 20889-5607, USA
e-mail: hoffmans@nmripo.nmri.nmhc.navy.mil*

William H. Bancroft

*Military Infectious Disease Research Program,
US Army Medical Research and
Material Command,
Frederick, Maryland 21703, USA*

Michael Gottlieb

*Parasitology and International Programs Branch,
NIAID/NIH,
Bethesda, Maryland 20892, USA*

Enriqueta C. Bond Burroughs

*Wellcome Fund,
Morrisville, North Carolina 27560, USA*

John R. Stephenson

Michael J. Morgan

*The Wellcome Trust,
London NW1 2BE, UK*

NERC maligned

Sir— Tina Yates' statement (*Nature* **386**, 754; 1997) that directors within the Natural Environment Research Council (NERC) are not concerned to create "an environment and career structure within which scientists can flourish over the longer term" is wrong.

In the NERC Centre for Coastal and Marine Sciences (CCMS), we have recently offered open-ended employment to 24 out of 36 (66 per cent) fixed-term (that is, impermanent) staff of more than five years' standing on contract. Just four (11 per cent) of the contracts have been allowed to expire; the remainder have been renewed for later review. This action is consistent with both the spirit and the letter of NERC policy.

Further, at a time of severe pressure on our core budget, it amounts to an expression of confidence by institute directors in the role of young scientists in maintaining the scientific vigour of the

laboratories. Young research staff employed on contract have never been viewed by the CCMS laboratories as some sort of flexibility buffer. On the contrary, they are an essential part of the research life of any active laboratory, and we have welcomed the opportunity to give practical expression to this view through the recent NERC employment policy.

Brian L. Bayne

*(Director)
Centre for Coastal and Marine Sciences,
Prospect Place, West Hoe,
Plymouth PL1 3DH, UK*

Once upon a time...

Sir— None of the discussions I have seen about the US government's plans to spend \$4 billion a year maintaining the safety and reliability of its stockpile of nuclear weapons has pointed out that once upon a time a small group of

scientists with no prior experience in the field and using 1940s knowledge and technology and no computers at all managed in the space of about three years to design two completely different atomic bombs, both of which worked spectacularly on the first try.

How can it now require a huge experienced design staff and new computers and experimental devices worth billions to assure the continued availability of weapons already designed and well tested? Such a claim certainly does not seem to attribute very much competence to those in the field today.

The only people who need \$4 billion a year spent on stockpile stewardship are those in whose pockets those dollars will end up and the politicians who want their votes.

Bill Mixon

*14045 North Green Hills Loop,
Austin, Texas 78737, USA
e-mail: billmixon@worldnet.att.net*

Demise of the monograph

Sir — It seems that everyone laments the publish-or-perish syndrome afflicting the modern scientific community¹. An unfortunate consequence of the increasing emphasis placed by funding bodies on numbers of research papers rather than overall quality of research has been the demise of the monograph.

Rather than writing comprehensive, integrated works, scientists today are encouraged to split their output into minimum publishable units. These 'paperlets' are either difficult to understand by themselves or result in much repetition due to necessary references to related paperlets. So shorter papers do not save journal space, but instead contribute to the publication explosion plaguing editors¹. They also make things difficult for scientists, as the information is not available in a single comprehensive work but is scattered in several places.

Another equally important but less discussed factor leading to the demise of comprehensive research papers has been pressure from publishers and editors. More and more journals are imposing strict page limits: there are now almost no high-profile

journals with a general readership that publish monographs. In contrast, the number of leading journals that publish only short papers has increased. It is no longer worthwhile writing longer papers because of the lack of high-profile journals that will publish them.

Philosophical Transactions A and *B*, the world's oldest journals, have bravely defied this trend. In my area (systematic biology), *Phil. Trans. B* is now the only high-profile general outlet for lengthy primary research reports. Such papers must otherwise be diverted to specialist journals, such as the Linnean Society publications, or museum annals with limited readership.

The last bastion of the monograph is, however, about to fall: it has just been announced that from 1 July, *Phil. Trans. A* and *B* will no longer accept primary research papers. Instead, they will publish only reviews and theme volumes². Although the reasons were not specified, it is likely that the financial and logistical strain of processing and publishing monographs forced the shift. No doubt *Phil. Trans.* will continue to publish important and timely review papers and anthologies. But this proposed niche is already occupied by several journals, such as *Biological Reviews*, *Quarterly Review of Biology* and *Annual Review of Ecology and Systematics*. From July, the unique role *Phil. Trans. A* and *B*

now perform for the scientific community (as two of the few remaining high-profile periodicals publishing detailed empirical papers of lasting importance) will end. And with fewer influential outlets for the comprehensive research paper — which everyone agrees is preferable to a plethora of insubstantial paperlets — scientists will be discouraged more than ever from writing them.

Michael S. Y. Lee

*School of Biological Sciences,
University of Sydney, Sydney,
NSW 2006, Australia
e-mail: msylee@bio.usyd.edu.au*

1. Werner, Y. L. in *Scientific Information Transfer: The Editor's Role* (ed. Balaban, M.) 113–121 (Reidel, Dordrecht, 1978).

2. Wall, P. D. *Phil. Trans. R. Soc. B* **352**, 255 (1997).

Events at Jena institute

Sir — The News article about the Institute for Molecular Biotechnology (IMB) in Jena (*Nature* **385**, 761; 1997) reported on the detailed internal affairs of the institute in an incomplete and one-sided way. I should like to respond to some of the statements in the article which I consider to be misleading.

Peter Schuster returned to Vienna because his three-year leave of absence from his home university ended in February 1995, but the article refers only to Schuster's

complaints about bureaucracy at the IMB. He turned down a permanent professorship at the University of Jena, and the opportunity to continue as scientific director of the IMB after negotiations with his home university in Vienna.

On the other hand, because of the excellent research facilities at the IMB, Schuster did not want to step down from his position as head of the IMB's evolutionary biology group. Because the IMB valued his expertise as an evolutionary biologist, Schuster was given the opportunity to run the IMB group from Vienna, but the geographical distance proved too much and left a noticeable leadership gap in the group.

The article refers to our cooperation with industry as "source of conflict", but cooperation with industry is a declared goal of the IMB, which has a considerable number of project contracts with industrial partners.

I should also like to point out that IMB scientists have themselves contributed to the situation at our institute. Early in 1996, some scientists chose not to use one of the several routine channels of in-house discussion, but instead complained anonymously to the press. Group leaders complained about the decision of the scientific advisory board, headed by Professor Rudolf Rigler, to keep evaluation reports confidential,

although this is normal procedure.

The contracts of two of Schuster's senior scientists ended in the autumn of 1996 and were supposed to be extended, assuming positive results from a pending evaluation of Schuster's group. Only after the scientists had appealed to the IMB's Kuratorium (supervisory board) and had made public complaints, including serious accusations against both directors of the IMB, did the scientific director decide not to renew their contracts.

As a result, the reputation of the IMB has been severely damaged, first by scientists of the IMB, including Schuster, who made their problems public before attempting to solve them in-house, second by lack of agreement of the chairman of the scientific council with members of the Kuratorium and the institute's directors, and last but not least by the publication of magazine articles.

Now that Schuster and Rigler have resigned, the IMB scientists can return to their proper task and, by using the outstanding technical facilities and funding at the IMB, can convince the scientific community of their quality by their achievements in research and development.

Stephan Diekmann

*Institute for Molecular Biotechnology,
Beutenbergstr. 11,
D-07745 Jena, Germany*

Biology, not microbiology

Sir — Contrary to what Kent, Scott-Ram and Thomas say (*Nature* **386**, 641; 1997), European patent law does not exclude "biological materials derived from 'essentially microbiological processes'". The European patent law, in its unfortunate language in Article 53 (b) EPC, expressly confirms the patentability of "microbiological processes or the products thereof".

Volker Vossius

*Holbeinstr. 5,
D-81679 München,
Germany
e-mail: dr.volker.vossius@t-online.de*

Scott-Ram replies — The sentence should have referred to "biological processes". The point that we were trying to make, and which still stands, is that one cannot "make use of a microbiological process in obtaining plants or animals only in order to be able to patent them".

Nick Scott-Ram

*British Biotech Pharmaceuticals Ltd,
Watlington Road,
Oxford OX4 5LY, UK*

Bless the baby! What a wally he has a-made!



Cause and Effect

The 'piddling school' of geology

Sir — As a contribution to the heated controversies that enlivened geology in its golden age in the first half of the nineteenth century, a number of satirical sketches and cartoons were drawn by Henry De la Beche (1796–1855), one of the leading geologists of his day and the founder of the Geological Survey of Great Britain. Several of these have been reproduced by Paul McCartney in his informative short biography¹ of De la Beche, but he believed that the one reproduced here had not survived. Happily, however, it has been preserved in the archives of the University Museum, Oxford, among the papers of William Buckland, the first Professor of Geology at Oxford.

The sketch (entitled "Cause and Effect") shows a toddler (said to represent Frank Buckland, William's eldest son) urinating at the head of a huge valley, with the nurse's comment "Bless the baby! What a wally he have a-made!". This was intended as an ironic comment on the uniformitarian outlook of Charles Lyell (a former pupil of Buckland, and Darwin's geological mentor), who championed the idea (now accepted for most valleys) that slow-acting erosion by rivers has formed the valleys they occupy, as opposed to pre-formation by earthquakes, faults, catastrophic floods or marine action. One of the objections to this so-called fluvial theory was provided by 'mis-fit' streams — huge valleys occupied by small rivers that it would seem could not have excavated them, even given an

immense period of time. It is this type of stream that De la Beche illustrates.

He evidently drew his picture at the time of the publication of Lyell's epoch-making *Principles of Geology* (1830–33), and it probably had wide circulation among geologists, although it was never published, perhaps being regarded as too improper for the time; doubtless Buckland made great play with it in his well-attended Oxford lectures. It evidently delighted Roderick Murchison, a stringent critic of Lyell; twenty years later, Murchison complimented De la Beche (whom he was soon to succeed as director-general of the Geological Survey) on the appearance of his massive volume *The Geological Observer*, but he added: "I could only have wished that it did not seem to me that you favoured the 'piddling' school more than of old, when you drew Frank Buckland as a baby denuding a valley. We shall hear a good deal more made of the book [*The Geological Observer*] tomorrow night; so I reserve my say except to hope that you have not actually become an inch by inch geologist." Murchison's comments of 1851 show that (unlike De la Beche) he had failed to come to terms with the concepts of deep-time (indications that the Earth is millions instead of thousands of years old) permitting the explanation of features of the Earth's surface by the cumulative effects of slow-acting processes as observed today, rather than by catastrophic events. The tiny stream in the huge U-shaped valley depicted by De la Beche suggests (with hindsight) excavation by a former mountain glacier — and by 1851 this possibility was already being accepted,

following the introduction of the 'glacial theory' to Britain by Buckland and Agassiz in 1840. Many geologists, however, found the idea that much of the Northern Hemisphere had been covered by immense ice sheets too fantastic, and Lyell, for one, never came to terms with such a neo-catastrophic event, which had no provable cause.

Why bother with such ancient controversies: are they not now dead and buried, and do we not understand the truth about the arguments? Apart from the interest in studying how our forebears, by painstaking data collection and flashes of inspiration and lateral thinking, laid the foundations of our modern world view, echoes of these (only) 150-year-old disputes are still with us. Although the Quaternary ice ages are accepted facts, there is still no universally accepted explanation of their origin; after a long period of fundamentalist uniformitarianism (the 'piddling school' of Murchison), invocation of impacts by comets and meteorites to explain global extinctions are now respectable; and punctuated evolution versus gradualism is still an issue. While we may not regret the decline in polemical quarrels over scientific theories, it seems a pity that we do not see more today of the satire and pictorial humour such as that from De la Beche and his contemporaries.

N. S. Haile

13 Talbot Road,
Oxford OX2 8LL, UK
e-mail: p0068328@brookes.ac.uk

1. McCartney, P. J. *Henry De la Beche: Observations on an Observer*. Friends of the National Museum of Wales, 1977.

The Earth's secret companion

Carl D. Murray

The Earth has a second companion — an asteroid, about 5 km across, in a peculiar type of orbital resonance called an overlapping horseshoe. It is probably only a temporary liaison.

Although sharing an orbit with a planet may seem an unlikely way for an asteroid to avoid collisions, being placed at the eye of the dynamical storm does offer some form of protection. The discovery by Wiegert *et al.* (see page 685 of this issue¹) that the unnamed asteroid 3753 shares its orbit with the Earth is the latest and most unusual example of a co-orbital resonant configuration that has been known for more than two hundred years.

The Italian–French mathematician Joseph-Louis Lagrange (1736–1813) studied the problem of three bodies moving under their mutual newtonian gravitational attraction. In 1772 he showed that a system consisting of the Sun and a planet had two equilibrium points located in the same orbit as the planet, one leading it by 60° and the other trailing by 60°, each forming an equilateral triangle with the two masses (Fig. 1). Lagrange showed that objects placed at these points were stable to small displacements provided the planet–Sun mass ratio was less than 0.04, and he predicted that Jupiter might harbour material at its leading and trailing points. It was not until the detection of 588 Achilles in 1906 that Lagrange's prediction was finally verified. There are now known to be several hundred such objects moving around Jupiter's triangular points, referred to as the Trojan asteroids. Not only was the type of orbital behaviour envisaged by Lagrange mathematically possible, but nature had obligingly provided actual physical examples.

Any elliptical orbit is characterized by its eccentricity (the degree of departure from a circle) and its semimajor axis (half the length of the orbit's long axis). Trojan asteroids have semimajor axes that are comparable to Jupiter's, and therefore they are special examples of resonant motion where the orbital periods of both objects are in the approximate ratio of 1:1. It is customary to consider the orbital paths of such objects in a frame rotating with the angular velocity of Jupiter. In this frame the Trojan orbits look like tadpoles, with large heads and elongated tails (Fig. 1). At any given time the asteroid is always moving on an ellipse around the Sun, but the orbit slowly evolves as a result of jovian perturbations.

The fact that the trailing and leading

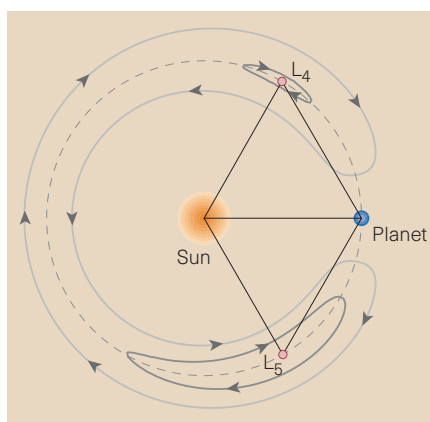


Figure 1 Three resonant orbits in a Sun–planet system, shown in a frame rotating with the planet's orbit. The stable equilibrium point L_4 lies ahead of the planet by 60°; the L_5 point trails. By Kepler's third law, those objects exterior to the planet move more slowly than it, and those interior move faster; that determines the direction of the path in the rotating frame. Here, two tadpole orbits are shown, as is a horseshoe orbit around both stable points. These are the actual paths traced out over many orbital periods in the case where the keplerian orbits are nearly circular. For eccentric orbits each path develops loops, as the object moves around its ellipse over one orbital period. Relative to the Earth, asteroid 3753 moves in an 'overlapping horseshoe' path (see Fig. 1 on page 685). Its orbit can overlap Earth's with no danger of collision because it is in a highly inclined plane.

points are stable for every planet orbiting the Sun and every satellite orbiting a planet (with the exception of the Pluto–Charon system) means that tadpole orbits could exist in other systems. This has proved to be so. In 1990 the asteroid (5261) Eureka was discovered orbiting in a 1:1 resonance near the trailing point of the Sun–Mars system². In Saturn's system, it is known that the moon Tethys has smaller moons orbiting close to each of its triangular points, and Dione shares its orbit with a single moon near its leading equilibrium point.

However, another type of orbit is possible in the 1:1 resonance. For a large enough difference between the semimajor axes of the planet and companion, the companion's orbital path can encompass both equilibri-

um points. The resulting path is referred to as a 'horseshoe' orbit (Fig. 1). The first example of a horseshoe configuration was announced in 1980 when Janus and Epimetheus, two newly discovered moons of Saturn, were found to be moving in orbits with a radial separation comparable to their smallest dimension (~50 km). The mass of Epimetheus is 25 per cent that of Janus and so they are large enough to perturb each other's motion. Every four years they encounter without passing, at which time Janus's semimajor axis changes by 10 km while that of Epimetheus changes in the other direction by 40 km, the situation reversing itself at the next encounter³. Once again, nature provided physical confirmation of a theoretical possibility.

The discovery that asteroid 3753 moves in a horseshoe orbit with respect to Earth could only have been made using the sort of numerical integration of the equations of motion carried out by Wiegert and colleagues¹. The true nature of its orbit was disguised by two things: the orbit is highly inclined to the plane of the Earth's orbit, and its large eccentricity gives rise to the 'loops' characteristic of eccentric paths in rotating frames (these are a simple consequence of viewing the asteroid's motion around the Sun from a frame rotating with the Earth's angular velocity). But the tell-tale switching of 3753's semimajor axis from one side of 1 AU to the other (that is, from inside to outside Earth's orbit) is a property of a horseshoe orbit that is more difficult to hide; 1 AU is the average distance between the Earth and Sun.

There are no simple guarantees of stability for any of the objects known to be in tadpole or horseshoe orbits, and the future for 3753 looks particularly bleak, with a chaotic orbit that can cross that of the Earth and Venus, while also coming close to the orbits of Mercury and Mars. The typical lifetime of such an object⁴ is expected to be about 100 million years.

But where do co-orbital objects come from and why are they not more prevalent, given that the stability condition is so easily satisfied? Whereas it is likely that 3753 is a temporary occupant of Earth's co-orbital niche, the Trojan asteroids in Jupiter's orbit are probably the tadpole survivors of a larger primordial population that once included some horseshoe orbits. Studies of test particles placed near Saturn's equilibrium points⁵ show that instabilities can arise when jovian perturbations are included. In the case of satellites of the outer planets, co-orbital objects could be fragments of the collisional break-up of an earlier, parent satellite. The dynamics of such processes are also applicable to the study of planetary rings, where embedded satellites that maintain particles in horseshoe orbits have been proposed to account for narrow rings⁶.

Asteroid 3753 won't hit the Earth soon, and we may well avoid physical contact with this particular 5-km companion indefinitely. Many puzzles remain, however, concerning the origin and evolution of all the co-orbital systems. At least now there is a growing realization among planetary scientists that an improved understanding of these intimate 1:1 relationships is essential to studies ranging from planetary accretion to ring dynamics. □

Carl D. Murray is in the Astronomy Unit, Queen Mary and Westfield College, London E1 4NS, UK.

1. Wiegert, P. A., Innanen, K. A. & Mikkola, S. *Nature* **387**, 685–686 (1997).
2. Mikkola, S., Innanen, K. A., Muinonen, K. & Bowell, E. *Celest. Mech. Dyn. Astron.* **58**, 53–64 (1993).
3. Nicholson, P. D. *et al.* *Icarus* **100**, 464–484 (1992).
4. Bottke, W. F. *et al.* in *Hazards due to Comets and Asteroids* (ed. Gehrels, T.) 337–357 (Univ. Arizona Press, Tucson, 1994).
5. de la Barre, C. M., Kaula, W. M. & Varadi, F. *Icarus* **121**, 88–113 (1996).
6. Dermott, S. F., Gold, T. & Sinclair, A. T. *Astron. J.* **84**, 1225–1234 (1979).

Human genetics

A father's imprint on his daughter's thinking

Peter McGuffin and Jane Scourfield

One of the puzzles of human gender differences is why boys are more vulnerable than girls to developmental disorders — such as autism — that affect language and social functioning. There has long been a suspicion that this could, in part, be explained by a more general sex difference, whereby women are usually better than men at those aspects of cognition that enable smooth social interactions ('social cognition'). Although such differences might be acquired, and reflect differing male and female social roles, they might also be part of the in-built biological dimorphism that results from the genetic difference between males and females. This intriguing possibility is explored by Skuse and colleagues¹ on page 705 of this issue, in a study of cognitive function in Turner's syndrome — a sporadic chromosomal disorder in which all (or part) of one X chromosome is missing. Patients have a female appearance and, usually, normal levels of general intelligence, but they have poorly developed secondary sexual characteristics, and short stature.

Normal females have two X chromosomes, and normal males have one X and one Y chromosome. But whereas females inherit one X chromosome from each of their parents, males always receive their X chromosome from their mother (Fig. 1). This means that, as well as the different chromosomal constitution, there is another possible source of male–female differences resulting from the phenomenon known as genomic imprinting². This refers to the finding that the same genes can result in different phenotypes (expressed characteristics) depending on whether they are inherited from the mother or from the father.

Although the mechanism of imprinting is not fully understood, part of the process involves methylation of DNA, which can effectively result in a gene from one or other parent being 'switched off'. To date there have been no reports of imprinting on the human X chromosome, but the phenome-

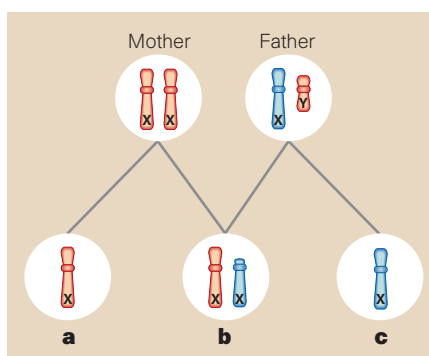


Figure 1 Females with Turner's syndrome have only one complete X chromosome, which may have come either from their mother (a) or from their father (c). They may also have deletions of part of the X chromosome, for example the short arm of the paternally derived chromosome (b). Skuse *et al.*¹ studied these patients, and showed that Turner's syndrome females with a paternally derived X chromosome (X^P) have much better social skills than those with a maternally derived X (X^M). They conclude that social functioning is influenced by an imprinted gene on the X chromosome that is switched off when the gene is inherited from the mother. And they believe that this may explain male–female differences, such as why men (who only have a maternally derived X chromosome) are more susceptible to developmental disorders that affect language and social functioning.

non does occur in other mammals such as the mouse³. Skuse *et al.*¹ have now tested the hypothesis that imprinting occurs at a gene that influences social cognition by comparing women with Turner's syndrome, whose single X chromosome came from their father, with those whose X chromosome was derived from their mother. In doing so, they have identified the first known case of imprinting on the human X chromosome.

Despite having normal intelligence, females with Turner's syndrome tend to show social difficulties⁴ — among the Turner's syndrome girls of school age who were

studied by Skuse *et al.*, many had been 'state-mented' (the official British system of noting special educational needs). But considerably more of the girls whose X chromosome was maternally derived (X^M) were in this category than those who had received their X chromosome from their father (X^P). Moreover, the X^M girls were judged to have more clinically significant social difficulties, such as offensive or disruptive behaviour, than the X^P girls or normal males and females.

The differences were explored further using a battery of psychological tests, including a newly devised parental-report scale to measure social cognition in their offspring. This showed that the X^P females had much better social cognition than the X^M patients, although both groups fared less well than normal (XX) female controls. The X^P females also had better verbal IQ than the X^M patients, but the authors suggest that the differences in social cognition and behaviour might be more closely related to subtle differences in cognitive ability that are not normally measured by conventional intelligence tests. In particular, there were significant differences between X^P and X^M patients in an attribute called 'behavioural inhibition'. This is measured by a set of tests in which selecting an appropriate response requires the concurrent inhibition or suppression of an inappropriate response. On the behavioural-inhibition task, X^P females had similar average scores to normal females, who in turn scored better than normal males.

Skuse *et al.* did a further set of experiments on a small group of patients in whom only part of the short arm of the paternally derived X chromosome was missing. In such cases, the partially deleted chromosome is usually inactivated. However, these women obtained similar scores in the social dysfunction tests to the Turner's syndrome X^P patients. These data indicate that there is an imprinted gene on the X chromosome that influences social functioning and related cognitive abilities. This gene escapes X-chromosome inactivation, and it is located either on the long arm of the chromosome, or on a small region of the short arm near the centromere. Such a gene can partly explain the male–female differences in social cognition, as well as in susceptibility to developmental disorders such as autism. But it does not provide the whole story, which is likely to involve an interplay between many genes and interacting environmental factors⁵.

This research raises two important issues, both of which are controversial. The first concerns the more general causes of differences in behaviour between the sexes. This includes both normal behaviour, such as different gender roles, and disorders such as depression (more common in women) and alcoholism (more common in men). Throughout most of the second half of this

century, with the increasing emphasis on sexual equality, there has been a tendency to play down the possible role of biology in accounting for psychological differences between men and women. Now, for the first time, we have evidence about the location of a gene that plays a part in behavioural sexual dimorphism, challenging the prevailing belief that gender differences are largely culturally determined.

The second controversial issue relates to the kind of behaviour that this gene influences. Skuse and colleagues¹ call it *social cognition*, but they show that it is associated with other aspects of cognitive ability. This raises the very real possibility that genes contributing to more commonly studied types of cognitive ability, such as those measured by IQ tests, will also soon be localized and eventually identified. Genetic research on IQ has often provoked hostility, not least because it has been open to political misuse⁶. Nevertheless, the evidence is consistent and

cognitive ability seems to be substantially heritable⁵. As for other aspects of behaviour, it is likely that each contributing gene will have only a small effect on the total population variation, which almost certainly involves many genetic and environmental factors. So the geneticist wishing to dissect out the molecular basis of behaviour needs to be prepared not just for controversy, but also for complexity. □

Peter McGuffin and Jane Scourfield are in the Division of Psychological Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, UK.

e-mail: mcguffin@cardiff.ac.uk

1. Skuse, D. H. *et al.* *Nature* **387**, 705–708 (1997).
2. Hall, J. G. *Am. J. Hum. Genet.* **46**, 857–863 (1990).
3. Zuccotti, M. & Monk, M. *Nature Genet.* **9**, 316–320 (1995).
4. McCauley, E., Ito, J. & Key, T. J. *Am. Acad. Child Psychiat.* **25**, 108–112 (1986).
5. Plomin, R., Owen, M. J. & McGuffin, P. *Science* **264**, 1733–1739 (1994).
6. Kamin, I. J. *The Science and Politics of IQ* (Wiley, Chichester, 1974).

Lower mantle

Aluminium under the spotlight

Jean-Paul Poirier

The deep interior of the Earth retains many secrets, and geophysicists and geochemists still hold conflicting views about some of its features. There is, however, one point on which everybody seems to agree: the lower mantle, between the depths of 670 and 2,900 km, essentially consists of an assemblage of crystals of magnesium and iron oxide, with sodium chloride structure (magnesiowüstite), and of magnesium and iron silicate, with perovskite structure.

Aluminium is abundant in the Earth's crust. But it is a minor element in the mantle, and in that context has been practically ignored. Now, however, in a paper on page 694 of this issue¹, Catherine McCammon reports results that should lead us to reconsider the importance of aluminium in the lower mantle. Using Mössbauer spectroscopy, she found that, although iron is normally present mostly as Fe^{2+} ions in the perovskite silicate, the proportion of Fe^{3+} may increase to about 50% in perovskite containing as little as 3.3 mol% Al_2O_3 . At first sight, this may not seem to be an Earth-shaking discovery, but the consequences for our understanding of the dynamics of the lower mantle (as well as for the chemistry of perovskites) could be momentous.

Perovskite structure is one of the most widespread crystal structures for compounds with the formula ABO_3 . It can be simply described as a tridimensional framework of corner-linked octahedra of oxygen atoms (Fig. 1). A cation of species B is nested inside every octahedron, while cations of

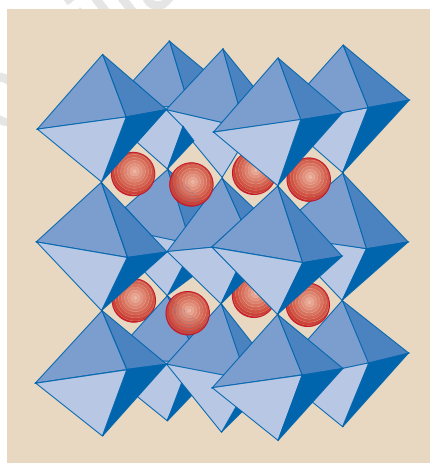


Figure 1 Crystal structure of the silicate perovskite: silicon atoms are nested inside oxygen octahedra (in blue; the oxygen atoms, not represented, are at the corners of the octahedra). The divalent cations Mg^{2+} or Fe^{2+} are represented as balls (red) between the octahedra. McCammon's results¹ suggest that Al^{3+} and Fe^{3+} cations may substitute for silicon or Fe^{2+} , and in consequence affect the properties, especially the electrical conductivity, of the lower mantle.

species A fit in dodecahedral sites between octahedra. Various distortions of the framework are possible, imparting different physical properties to the crystals. In the lower-mantle silicate perovskite $(\text{Mg,Fe})\text{SiO}_3$, which is stable only at pressures higher than about 25 GPa, Si^{4+} ions are inside the octahedra and Mg^{2+} and Fe^{2+} ions are randomly distributed between octahedra.

Two papers on aluminium^{2,3} had already attracted the attention of the mineral-physics community. In laser-heated diamond-anvil cell experiments, Kesson *et al.*² were able to synthesize silicate perovskite containing up to 25 mol% Al_2O_3 . They demonstrated that Al_2O_3 in solid solution could be accommodated in perovskite and that there was no need for new, unknown, aluminous minerals in the lower mantle. Iron tends to destabilize perovskite, so that perovskites with a high iron content do not exist. However, Kesson *et al.* showed that Al_2O_3 extends the stability field of magnesian perovskite towards iron-rich compositions, up to 75% atom% Fe.

The lower-mantle assemblage of magnesiowüstite and perovskite results from the disproportionation of $(\text{Mg,Fe})_2\text{SiO}_4$ and other silicates, and it has been experimentally verified that iron preferably partitions into magnesiowüstite. In the other recent paper on aluminium, Wood and Rubie³ have shown that, in the presence of Al_2O_3 , perovskite and magnesiowüstite have similar values of the $\text{Fe}/(\text{Fe}+\text{Mg})$ ratio.

So it seems to be established that aluminium controls the iron content of perovskite. McCammon's results¹ take us a step further and raise exciting new problems. Iron is a transition metal, and can take two degrees of oxidation: Fe^{2+} and Fe^{3+} , the ferrous and ferric forms, respectively. McCammon availed herself of the fact that iron is one of the few elements amenable to Mössbauer spectroscopy to determine its degree of oxidation in perovskite containing aluminium. Whereas iron is mostly present as Fe^{2+} in aluminium-free perovskite, the relative amount of Fe^{3+} significantly increases to about 50% in the presence of aluminium.

McCammon's data suggest that Fe^{3+} substitutes for silicon in the octahedral sites as well as for Fe^{2+} in dodecahedral sites. The instability of iron-rich perovskites is usually ascribed to the large crystal-field energy of Fe^{2+} in dodecahedral sites. The presence of iron as Fe^{3+} in octahedral sites may not be subject to such restrictions. Al^{3+} might also occupy both sites. The sum of Al and Fe in octahedral and dodecahedral sites should balance for electrical neutrality. There would then be no need to introduce charge-compensating vacancies, as would be the case if Fe^{3+} only substituted for Fe^{2+} in dodecahedral sites. Quasi-chemical accounting of point defects, currently performed to investigate the various possible controls of diffusion, should not only take aluminium into account, but also consider where it sits in the lattice.

Fe^{3+} ions are much smaller than those of Fe^{2+} . Depending on which type of site they occupy, they may induce distortions of the framework of octahedra different from those in aluminium-free perovskite. Also, the lattice parameters may be different, hence the density and equation-of-state of

lower-mantle perovskite may not be quite the same as that of the experimentally determined, aluminium-free perovskite. As seismological models of the lower mantle undergo continual improvement, slight differences in density and elastic moduli may become relevant.

Finally, the most important consequences of the presence of a large amount of Fe^{3+} in the aluminous lower mantle may be in the field of transport properties, possibly viscosity, but surely electrical conductivity. Whether electromagnetic core–mantle coupling is possible or not depends on the electrical conductivity of the lower mantle. In recent years, experiments in diamond-anvil cells have led to the conclusion that conduc-

tivity is probably controlled by hopping of holes in the lattice from Fe^{3+} to Fe^{2+} ions⁴. So perovskite conductivity depends on Fe^{3+} concentration. Surely, the now distinct possibility that aluminium in the perovskite lattice induces a high concentration of Fe^{3+} is an incentive to start experiments on aluminous perovskites. □

Jean-Paul Poirier is in the Département des Géomatériaux, Institut de Physique du Globe de Paris, 4 place Jussieu, 75252 Paris Cedex 05, France. e-mail: poirier@ipgp.jussieu.fr

1. McCammon, C. *Nature* **387**, 694–696 (1997).
2. Kesson, S. E., FitzGerald, J. D., Shelley, J. M. G. & Withers, R. L. *Earth Planet. Sci. Lett.* **134**, 187–201 (1995).
3. Wood, B. J. & Rubie, D. C. *Science* **273**, 1522–1524 (1996).
4. Poirier, J. P., Goddat, A. & Peyronneau, J. *Phil. Trans. R. Soc. Lond.* **354**, 1361–1369 (1996).

Transcriptional activation

Something new to hang your HAT on

Marc Montminy

Someone once said, “Simplicity is making the journey... with just baggage enough”. For those interested in the complexities of gene regulation, characterizing the mechanisms by which nuclear factors engage the transcriptional apparatus in response to hormonal stimulation has seemed, at times, to be an insurmountable task. But on pages 677 and 733 of this issue, Torchia *et al.*¹ and Heery *et al.*² report the identification of a short peptide motif that mediates the assembly of nuclear receptor–co-activator complexes. This result has implications for understanding the mechanisms by which nuclear receptors ultimately interact with the transcriptional machinery in a signal-dependent manner.

The nuclear receptors are a superfamily of hormone-inducible transcription factors that regulate gene expression in response to small ligands such as steroid and thyroid hormones, retinoids and vitamin D. Organized in a modular fashion, the nuclear receptors contain discrete domains that are involved in DNA recognition, ligand binding, dimerization and transactivation³. Hormone binding stimulates nuclear-receptor activity, in part by promoting a conformational change in a helical transactivation domain that is known as AF2. This change occurs through an allosteric mechanism, and it is thought to promote interactions between AF2 and proteins in the transcriptional apparatus^{4,5}.

Because investigators have been unable to observe direct functional interactions between most nuclear receptors and so-called ‘general transcription factors’, they have recently tried to identify intermediary proteins — or co-activators — which could bridge the association between nuclear receptors and the transcriptional machinery. This search has led to the characterization of

the steroid-receptor co-activators (SRCs), which bind directly to the AF2 region of several nuclear hormone receptors^{6,7}.

But how do nuclear-receptor-bound SRCs, in turn, promote transcriptional activation? Enter CREB-binding protein (CBP). First characterized as a co-activator for genes that are responsive to cyclic AMP and mitogens^{8,9}, CBP has been shown to mediate transcriptional stimulation in response to a diverse array of cellular signals, including

steroid and thyroid hormones, and retinoids^{10,11}. CBP and its homologue P300 seem to stimulate the activity of a target gene through their intrinsic histone acetyltransferase activities^{12,13}, and through their association with functional RNA polymerase II complexes¹⁴ (Fig. 1).

The current view, then, is that SRC proteins mediate the activation of nuclear hormone receptors by virtue of their association with CBP and P300 (refs 15, 16). Now, Torchia *et al.*¹ show that a hitherto undiscovered member of the SRC-1 family — termed p/CIP — is important not only for the activation of nuclear receptors but, in contrast to other SRCs, also in the response to signalling by interferon- γ and phorbol esters. The functional distinction between p/CIP and the other SRC-family members is perplexing in view of their substantial sequence similarities, particularly in the nuclear-receptor and CBP-binding domains. The solution to this puzzle will probably have to wait for gene-disruption studies, in which cells lacking p/CIP or other SRCs can be compared for their response to hormones.

So how are these nuclear receptor–co-activator complexes assembled in response to hormonal signals? Torchia *et al.* and Heery *et al.* describe a short, leucine-rich motif (LXXLL; where L denotes leucine and X denotes any amino acid), found in the nuclear-receptor interaction domains of both SRC- and CBP-family members. This motif seems to mediate interaction with the

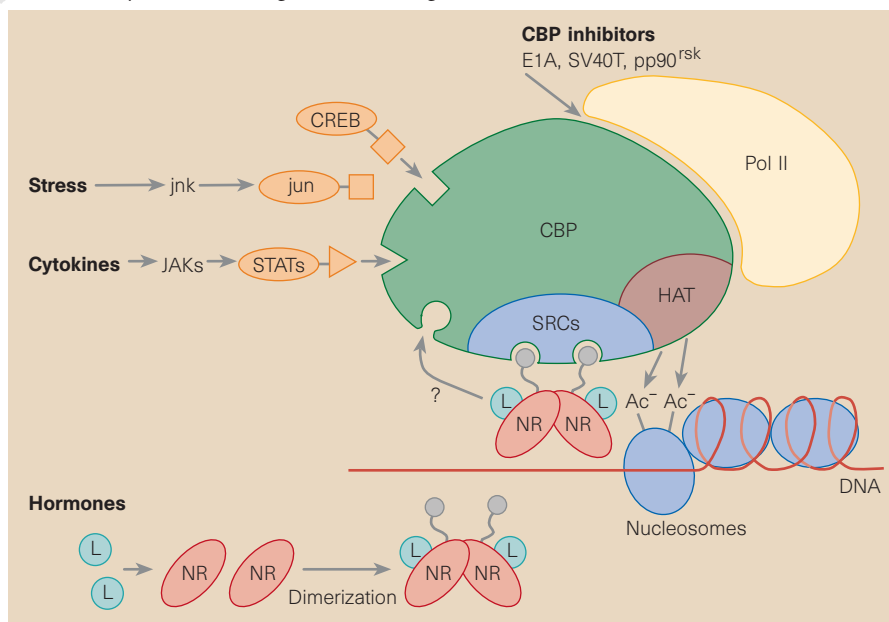


Figure 1 The CREB-binding protein (CBP) is a general mediator of signal-dependent transcription. In response to various stimuli, CBP associates with a variety of signal-dependent factors including CREB, jun, STATs 1 and 2, and nuclear hormone receptors (NR). CBP seems to be constitutively associated with the steroid-receptor co-activators (SRCs), which can also recognize ligand (L)-bound nuclear receptors. Torchia *et al.*¹ and Heery *et al.*² now show that assembly of these nuclear receptor–co-activator complexes is mediated through a short, leucine-rich motif, LXXLL. After CBP is recruited to signal-dependent promoters, it is thought to stimulate the expression of target genes through its association with RNA polymerase II complexes (Pol II), and through its intrinsic histone acetyltransferase (HAT) activities (the addition or removal of acetyl (Ac⁺) groups).

nuclear receptors. SRC-1, for example, contains four LXXLL motifs, which are essential for its interaction with the oestrogen receptor, and to mediate the induction of target genes in a ligand-dependent manner².

The involvement of leucine-rich motifs in the assembly of nuclear-factor complexes is not new. Leucine-zipper domains, for instance, have been shown to mediate dimerization of nuclear factors such as jun, fos and CREB, through a heptad repeat of leucine residues¹⁷. However, not all of the proteins that contain leucine zippers can form heterodimers with one another — partner specificity is determined by the presence of intervening polar residues, which form salt bridges to further stabilize homo- and heterodimer formation¹⁷.

Torchia *et al.* and Heery *et al.* support the notion that, in a similar manner, the residues within the LXXLL motifs may determine which nuclear receptors assemble with which co-activators following hormonal induction. An acidic amino acid at position 3 within the LXXLL motif, for example, seems to be particularly unfavourable for interaction with the oestrogen receptor². Notably, CBP contains an (acidic) glutamate residue at position 3 of its LXXLL motif, and seems to bind the AF2 domain of the oestrogen receptor far less efficiently than a motif in SRC-1 which contains a (basic) arginine residue at the same position². Given the apparently striking differences in the affinity of the oestrogen receptor for binding CBP and SRC1, it will be important to work out which

of these related motifs are used by individual nuclear-receptor family members *in vivo*.

Our understanding of the process by which steroid hormones and other extracellular signals stimulate target-gene expression is far from complete. But the description of a short motif that mediates the formation of complexes between nuclear receptors and these co-activators greatly simplifies that endeavour, by allowing for the eventual development of antagonists that can disrupt these complexes. □

Marc Montminy is in the Section on Molecular Biology, Joslin Diabetes Center, One Joslin Place, Boston, Massachusetts 02215, USA.

1. Torchia, J. *et al.* *Nature* **387**, 677–684 (1997).
2. Heery, D. M., Kalkhoven, E., Hoare, S. & Parker, M. G. *Nature* **387**, 733–736 (1997).
3. Tsai, M. J. & O'Malley, B. W. *Annu. Rev. Biochem.* **63**, 451–486 (1994).
4. Danielian, P. S., White, R., Lees, J. A. & Parker, M. G. *EMBO J.* **11**, 1025–1033 (1992).
5. Durand, B. *et al.* *EMBO J.* **13**, 5370–5382 (1994).
6. Onate, S. A., Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. *Science* **270**, 1354–1357 (1995).
7. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P. & Gronemeyer, H. *EMBO J.* **15**, 3667–3675 (1996).
8. Kwok, R. P. *et al.* *Nature* **370**, 223–226 (1994).
9. Arias, J. *et al.* *Nature* **370**, 226–229 (1994).
10. Kamei, Y. *et al.* *Cell* **85**, 403–415 (1996).
11. Chakravarti, D. *et al.* *Nature* **383**, 99–103 (1996).
12. Ogryzko, V. V., Shiltz, R. L., Russanova, V., Howard, B. H. & Nakatani, Y. *Cell* **87**, 953–960 (1996).
13. Bannister, A. J. & Kouzarides, T. *Nature* **384**, 641–643 (1996).
14. Nakajima, T., Uchida, C., Anderson, S. F., Parvin, J. D. & Montminy, M. *Genes Dev.* **11**, 738–747 (1997).
15. Yao, T. P., Ku, G., Zhou, N., Scully, R. & Livingston, D. M. *Proc. Natl Acad. Sci. USA* **93**, 10626–10631 (1996).
16. Smith, C. L., Onate, S. A., Tsai, M. J. & O'Malley, B. W. *Proc. Natl Acad. Sci. USA* **93**, 8884–8888 (1996).
17. Landschulz, W. H., Johnson, P. F. & McKnight, S. L. *Science* **240**, 1759–1764 (1988).

Mathematics

The restless heart of a spiral

Arun V. Holden

Rotating spiral waves occur in a variety of two-dimensional systems that all share the property of excitability. A spiral wave has wave-like and particle-like properties — it pervades the medium and has a phase, but it also has a position, given by the coordinates of its tip. The motion of a spiral wave¹, defined by motion of its tip, is called meander. This is not just a mathematical curiosity: the potentially lethal re-entrant cardiac arrhythmias of flutter and fibrillation, for example, in which the same wave of excitation repeatedly re-invades the same piece of tissue, can be modelled as spiral waves. Meander is a mechanism by which such arrhythmias can stop; and the artificial control of meander offers a means of low-voltage cardiac defibrillation, by pushing the wave tip out of the heart. At a meeting in May*, an emerging mathematical understanding of meander was described that uses distance-preserving symmetries to simplify the analysis.

A full mathematical description of waves in an excitable medium requires the solution of a partial differential equation, in which the variables change continuously with space and time over the whole plane. A rigidly rotating spiral wave is a time-periodic solution, whose wavefront has a shape close to that of an archimedean spiral, and the outward propagation of the wavefront produces the rotation of the spiral. The tip of a rigidly rotating spiral propagates around a circular core, within which all is at rest. If the tip is not following a simple circle (see Fig. 1), the spiral wave is distorted.

If you rotate, reflect or translate a spiral, you still have a spiral. These distance-preserving transformations of the plane are the symmetries of the euclidean group E(2). Based on these symmetries, Barkley² introduced *ad hoc* an ordinary differential equation that describes spiral wave motion simply as motions of the tip (instead of variations over the whole plane). Solutions of this equation undergo a 'Hopf bifurcation', from a motion

with a single frequency (rigid rotation) to periodic motion with two frequencies (rotation plus meander). At resonance — that is, when the two frequencies are equal — the spiral follows a superposition of a linear drift and rigid rotation. The behaviour of this system closely matches that of spiral wave dynamics obtained as solutions of the full partial differential system², and seen in experiments³. Furthermore, the approach naturally leads to using external periodic perturbations (in the heart, an electrical signal) to induce a controlled movement of the spiral by resonant drift⁴.

But to confidently design a method for controlling cardiac arrhythmias in this way, we must do better than an *ad-hoc* model: a rigorous mathematical framework is needed to define the range of behaviours over which the method can be applied. A rigorous derivation means reducing the system so that its motion can be separated into a superposition of motion that preserves the E(2) symmetries (spiral properties), and motion 'across' the group — the tip meander, which lacks these symmetries.

Although such a reduction is routine for ordinary differential systems, there are severe difficulties in such an approach to the partial differential equations that generate meandering spiral waves. These formidable technical problems have now been resolved (C. Wulff, Univ. Berlin). A rigorous proof of the necessary reduction theorem has been achieved⁵, bifurcations of the reduced differential equations have been studied^{6,7}, and the evolution of single and multi-armed spirals in physical space explained⁷.

A simple geometrical interpretation of these rigorous results can be made for single-armed spiral waves⁸ (V. N. Biktashev, Univ. Leeds). The complicated dance of a meandering or drifting spiral wave can be simplified by moving to a rotating frame of reference attached to the tip of the spiral. This



Figure 1 Visualization of the spiral wave solution of the mammalian ventricular tissue model (reproduced from ref. 11). The white lines are voltage and Ca^{2+} activation variable isolines; the yellow ball defines the tip position by their intersection. The orange-yellow curves map the meandering tip trajectory over the two preceding rotations of the spin.

*Spiral Waves, Euclidean Symmetries and Cardiac Arrhythmias
Warwick, UK, 12 May 1997.

gives a system of ordinary differential equations describing the tip trajectory, or meaner, with the frame of reference preserving the spiral nature and euclidean symmetries.

So Barkley's model system has been derived rigorously. But the heart is a three-dimensional object with a complicated and moving geometry. Re-entrant waves are not spirals with a point tip, but scrolls, wrapped around a line filament. This filament can be curved; closed, as in a scroll ring, or open; twisted due to rotational anisotropy; and even under tension. Now that we have a rigorous basis for spiral wave motion, we can extend the analysis to a three-dimensional medium and consider motions of scroll waves that are possible within the $E(3)$ symmetry group⁹ (P. Ashwin, Univ. Surrey). For example, a simple scroll can rotate and drift, whereas a twisted scroll can only rotate, and a double scroll has to remain stationary. A simple scroll ring can drift along its axis, or expand.

Such motions are seen in numerical solutions of three-dimensional caricatures (sim-

plified models) of cardiac tissue, and are believed to occur during ventricular tachycardias¹⁰. Once a rigorous three-dimensional mathematical description of these excitations is available, we may finally be able to control real disturbances in the heart. □

Arun V. Holden is at the Centre for Nonlinear Studies, University of Leeds, Leeds LS2 9JT, UK.

e-mail: arun@cbl.leeds.ac.uk

1. Holden, A.V., Markus, M. & Othmer, H. G. (eds) *Nonlinear Wave Phenomena in Excitable Media* (Plenum, New York, 1991).
2. Barkley, D. *Phys. Rev. Lett.* **72**, 164–167 (1994).
3. Li, G., Ouyang, Q., Petrov, V. & Swinney, H. L. *Phys. Rev. Lett.* (in the press).
4. Mantel, R. M. & Barkley, D. *Phys. Rev. E* **54**, 4791–4801 (1996).
5. Sandstede, B., Scheel, A. & Wulff, C. J. *Diff. Eq.* (in the press).
6. Fiedler, B., Sandstede, B., Scheel, A. & Wulff, C. *Doc. Math. J. DMV* **1**, 479–505 (1996).
7. Golubitsky, M., LeBlanc, V. G. & Melbourne, I. *J. Nonlinear Sci.* (in the press).
8. Biktashev, V. N., Holden, A.V. & Nikolaev, E. V. *Int. J. Bifurcation Chaos* **6**, 2433–2440 (1996).
9. Ashwin, P. & Melbourne, I. *Nonlinearity* **10**, 595–616 (1997).
10. Panfilov, A. V. & Holden, A. V. (eds) *Computational Biology of the Heart* (Wiley, Chichester, 1997).
11. Biktashev, V. N. & Holden, A. V. *Proc. R. Soc. Lond. B* **263**, 1373–1382 (1996).

Reductionism

The ends of understanding

Paul Nurse

If we had a complete description of all the molecular reactions occurring within a living cell, would we 'understand' that cell? Scientists seldom give much thought to such questions, and so it was an unusual gathering that assembled last month* to discuss how far a reductionist approach can take biology. Reductionism seeks to explain the wide variety of natural phenomena by the behaviour of limited numbers of simpler constituents subject to rigorous and simple laws. It has been a powerful driving force in science and its success is plainly evident in the spectacular triumphs of molecular biology which have provided an understanding of the molecular basis of areas such as developmental biology, immunology and human disease.

However, as the sole philosopher present argued (T. Nagel, NYU Law School), there are limitations to the reductionist programme. It is unlikely to be useful to try and explain biological phenomena in terms of particle physics, even if they could be so reduced. There are other levels such as molecules, genes, cells, organisms and populations which are more appropriate for adequate explanations. But what, then, is meant by an 'adequate explanation', and what is the most appropriate level to which a phenomenon should be reduced? Moreover, some generalizations and theories developed in biology may require principles that are additional to, but not inconsistent with, physical laws. Examples are concepts such as a gene, a

wing or an instinct, and the theory of natural selection — these centre on function and purpose, which are only meaningful in biology, and not physics or chemistry.

One straightforward point that strikes me is the importance of using appropriate methodologies to explain the phenomenon of interest. Thus, when reducing phenomena of cell biology to molecules and their interactions, methodologies should be employed which study molecules in the real time and space of the living cell, otherwise much of the richness of cell behaviour may be lost. For example, growth-factor signalling is often described in terms of a simple on/off device, but important information can be conveyed in the dynamics of a signalling pathway in a manner analogous to the communication of messages by the sequence and duration of signals in Morse code. The various steps of a pathway could be represented as information-processing elements, such as amplifiers, timers or integrators, as is found in an electronic circuit, rather than in terms of molecular interactions. With such representations, the same machine logic as the process under study should be used, otherwise the modelling is merely a simulation rather than an explanation. However, the detail of the molecular interactions do need to be fully described when investigating or treating a pathology (K. Holmes, MPI für Medizinische Forschung, Heidelberg), although there can be difficulties if the whole process is not also considered — for example, treatment of cardiac arrhythmias using drugs that

interfere with specific molecular activities requires understanding of how the heart behaves as a whole (D. Noble, Univ. Oxford).

The importance of information processing in biology received further emphasis with the proposal that living organisms should be considered as analog computing machines which store information in the form of molecular structures (S. Brenner, Molecular Sciences Research Inst., La Jolla). Cells should not be considered as algorithmic calculating devices, but rather as a set of look-up tables from which information can be retrieved. Thus, the information specifying the Michaelis constant, K_m , of an enzyme is 'stored' in the protein structure which in turn is encoded within the structure of DNA, the ultimate repository of information. The mapping of phenotypes to specific genes is equivalent to realizing the programme encoded by the DNA, but such mapping is often very complex because it depends on the actions of other interacting genes and on the past history and environment of the organism.

There are especial difficulties with such mapping in behavioural studies. The proper study of mental processes requires consideration of the products of minds and of the interactions between minds (H. Barlow, Univ. Cambridge). These processes are not easily reducible to cellular and molecular behaviours. For instance, it could be imagined that the recognition of 'mother' by a chick may result in the stimulation of a specific set of ten particular neurons. If these neurons are cultured in a Petri dish and then treated in a way that mimics mother recognition, would these cells in any way be experiencing the concept of mother? This seems particularly absurd. In a similar vein, can the concept of being in love be made explicable in terms of neuronal activity? It is evident that appropriate understanding needs explanation at the appropriate level. Likewise in studies of evolution there has been a vigorous debate over the units of selection, including arguments for group selection, inclusive fitness and gene-centred approaches (J. Maynard Smith, Univ. Sussex).

What conclusions emerged from the meeting? Unsurprisingly, there was broad support for a pragmatic pluralist approach — that is, the study of phenomena at a variety of levels. Explanations in science must always have some elements of reductionism, but descriptions of increasing detail may only provide a delusion of understanding; overenthusiastic pursuit of reductionism can limit discovery and also has ideological implications in defining what is considered to be the best science in terms of publication and financial support (S. Rose, Open Univ., Milton Keynes). But a real problem that remains is what constitutes an 'adequate explanation'. Can this ever be more than a matter of individual taste? □

Paul Nurse is at the Imperial Cancer Research Fund, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.

* *The Limits of Reductionism in Biology*, Ciba Foundation, London, UK, 12–15 May 1997.



100 YEARS AGO

Nothing is worse than fog at sea. A storm may cause discomfort, an accident may cause delay, but in neither case does the traveller feel so helpless as when his vessel is completely shut in by a dense fog. To lessen the danger which then exists, Prof. E. C. Pickering, the Director of the Harvard College Observatory, suggests, in a pamphlet just received, a method of determining the position of a vessel in a fog, based upon the velocity of sound. If two fog-horns of different pitch be placed at equal distances from the middle of a channel or entrance to a harbour, and be sounded simultaneously at regular intervals of about a minute, it will be evident that a captain of a vessel will be able to locate his position with fair accuracy by noting when the sounds of the horns are heard. If the two sounds are heard at the same instant the vessel will be in the middle of the channel, and if they are heard after one another it would be possible to judge from the interval between the two how much the vessel is out of the middle of the channel.

From *Nature* 10 June 1897.

50 YEARS AGO

On June 2, 1897, in his well-known address to the Victoria Institute on "The Age of the Earth", Lord Kelvin said: "I must first ask you to excuse my giving you all my depths, heights, and distances, in terms of the kilometre, being about six-tenths of that very inconvenient measure the English statute mile, which with all the other monstrosities of our British metrical system, will, let us hope, not long survive the legislation of our present Parliamentary session destined to honour the sixty years' Jubilee of Queen Victoria's reign by legalising the French metrical system for the United Kingdom". The Weights and Measures (Metric System) Act was passed, and it is no longer a punishable offence for a tradesman to have in his possession a weight or measure of the decimal system. But with all the "monstrosities of our British metrical system" still surviving, it is interesting to recall the fiftieth anniversary of Lord Kelvin's unfulfilled hope. — 'Lord Kelvin and the British metrical system.'

From *Nature* 14 June 1947.

The Solar System

The frontier beyond Neptune

Glen R. Stewart

Until five years ago, Pluto was viewed as a lonely outpost at the edge of the Solar System. Then in September 1992, Dave Jewitt and Jane Luu¹ discovered the first of a population of small objects beyond Neptune. These objects, which make up what has come to be known as the Kuiper belt, are in nearly-circular orbits that have remained largely unchanged since the formation of the outer planets². In last week's *Nature*³, Luu *et al.* presented the discovery of a new type of trans-neptunian object, 1996TL₆₆. It has an unusually eccentric orbit with a very large semimajor axis, and is probably one of the first of a population of objects that were scattered into this region by Neptune. Its discovery marks an important stage in our understanding of planet formation and the structure of the outer Solar System, and apparently confirms the existence of a 'scattered disk' — a new component of the Solar System predicted by Duncan and Levison in tomorrow's *Science*⁴.

Although speculation on the existence of the Kuiper belt dates back almost 50 years^{5,6}, serious work on the topic did not start until Fernández⁷ invoked it as a possible source of the short-period comets (those with orbital periods of less than 200 years). Dynamical simulations⁸ showed that a comet belt beyond Neptune is the most plausible source for the low-inclination, Jupiter-family comets. The first Kuiper-belt object, an icy body about 250 km across, was discovered in 1992.

So far, 40 Kuiper-belt objects have been

discovered with ground-based telescopes. As only a small fraction of the sky has been searched, these 40 imply⁹ a total population of 100-km objects of about 70,000, assuming that most objects are near the ecliptic (the plane of the Earth's orbit). Of the 40 known objects, 21 have well-determined orbits, and fall into two main classes. Inside 41 AU (Neptune is at 30 AU), they all have eccentricities between 0.1 and 0.3, and they are in mean-motion resonances with Neptune — that is, the ratio of their orbital period to Neptune's is a ratio of small integers. Beyond ~41 AU, they do not tend to be in mean-motion resonances and mostly have eccentricities less than 0.1. All the well-observed Kuiper-belt objects have semimajor axes of less than 50 AU and inclinations less than 20°.

To explain this surprisingly intricate structure, models have included the effects of the migration of Uranus and Neptune as they formed¹⁰, the effects of a massive protoplanetary disk¹¹, and the effects of individual massive planetesimals¹² on the orbits of Kuiper-belt objects. These models predict that Kuiper-belt objects will have eccentricities in the range 0–0.3 and inclinations of 0–15°.

The new prediction of a 'scattered disk' comes from simulations⁴ of the behaviour of objects that originated in the region between Uranus and Neptune or that were perturbed by Neptune from the inner edge of the Kuiper belt. Some of these objects can come close to Neptune and get scattered into eccentric orbits outside its orbit. The long-

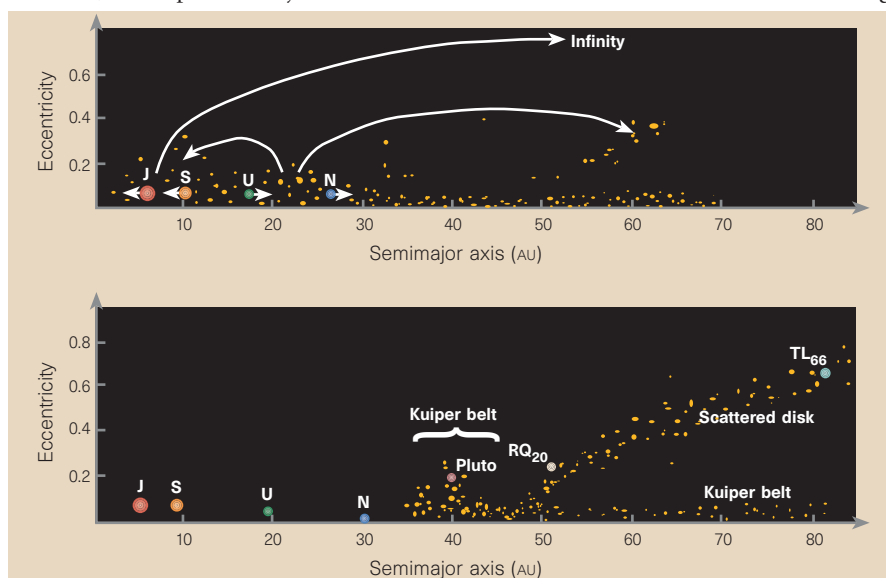


Figure 1 Creation of the scattered disk. Jupiter and Saturn can scatter nearby comets out of the Solar System, but Uranus and Neptune are gentler, and send some comets into long-lived orbits with high eccentricity and semimajor axis. The newly discovered 1996TL₆₆ may be a member of this scattered disk. (Adapted from ref. 4.)

range gravitational effects of the planets can lift the orbits of these objects beyond the outer edge of the planetary system, protecting them from close encounters with the planets. Therefore, many of these objects can survive in these eccentric orbits for the 4.6-billion-year age of the Solar System, forming a scattered disk.

Duncan and Levison's numerical simulations⁴ indicate that objects in the scattered disk tend to have more eccentric and/or inclined orbits than those in the Kuiper belt. Two recently discovered trans-neptunian objects may be the first members of the scattered disk to be found. The first, 1996RQ₂₀, was discovered in September 1996 by E. Helin, D. Brown and D. Rabinowitz. Its semi-major axis is 47 AU, its eccentricity is 0.3, its inclination is 32°, and it is about 300 km across¹³. The newly discovered second object, 1996TL₆₆, is estimated³ to have a semimajor axis of 84 AU, an eccentricity of 0.58, an inclination of 24°, and to be about 490 km across. These objects are clearly outside the range of orbital elements predicted for the Kuiper belt: 1996RQ₂₀ because of its inclination, and 1996TL₆₆ because of its eccentricity. However, they could be members of the scattered disk⁴.

With the recent advances in our observational and theoretical understanding of the trans-neptunian region, it is now possible to construct a tentative picture of how the outer Solar System formed and evolved.

Jupiter and Saturn formed early on, when the gaseous component of the solar nebula was still present. As the gas dissipated, it left behind a large number of small, icy bodies that had condensed from it, outside the orbit of Saturn and extending outward perhaps as far as a few hundred AU. These objects collided and gradually grew into larger 'planetesimals'. As the planetesimals increased in mass, they began to gravitationally perturb one another into more eccentric and more inclined orbits about the Sun. The growth of planets was much more rapid inside about 40 AU, leading to the formation of Uranus and Neptune. But outside 40 AU (that is, in the Kuiper belt), the bodies remained relatively small. Pluto may be just the largest Kuiper-belt object.

The young Uranus and Neptune scattered most of the remaining nearby planetesimals, outwards into the trans-neptunian region and inwards towards Jupiter and Saturn (Fig. 1). Usually, the objects scattered outwards eventually returned to the Uranus–Neptune region. In contrast, Jupiter and Saturn are so massive that they can efficiently eject many of these planetesimals out of the planetary system — so there was a net flux of objects towards Jupiter. This inward transport required an outward migration of the orbits of Uranus and Neptune in order to conserve the angular momentum and energy of the system¹⁴. As

Neptune moved outwards, it may have trapped planetesimals farther out into mean motion resonances¹⁰. Then Neptune's migration (and other processes^{11,12}) would have pumped up the eccentricity of these objects to values as large as 0.3, leading to much of the present Kuiper-belt structure described above.

As mentioned, the objects scattered outwards by Uranus and Neptune can be long-lived. After their first encounter with Neptune, about 1% of them are stored in an extended scattered disk beyond Neptune for the age of the Solar System⁴. There are 31 Earth-masses of material in Uranus and Neptune, and as planet formation is far from 100% efficient, it is reasonable to expect that a similar amount of mass was initially scattered outwards by Neptune (the efficiency factor is not known to better than a factor of a couple of orders of magnitude). So, crudely, we may expect as much as a few tenths of Earth-masses of material in the scattered disk. This value is consistent with current estimates based on the dynamical models of the scattered disk by Duncan and Levison.

Much of the above picture is speculative, and much of it is likely to change rapidly in the coming years or months as this field

matures and more data become available. One reason for this is that only a very small fraction (about 0.06%) of the Kuiper-belt and scattered-disk objects have yet been discovered. But the fact remains that the structure of the trans-neptunian region is much more complex, and therefore interesting, than was dreamed of only a few years ago. □

Glen R. Stewart is in the Laboratory for Atmospheric and Space Physics, University of Colorado, Boulder, Colorado 80309, USA.
e-mail: glen@ptah.colorado.edu

1. Jewitt, D. & Luu, J. *Nature* **362**, 730–732 (1993).
2. Weissman, P. R. & Levison, H. F. in *Pluto* (eds Stern, S. A. & Tholen, D. J.) (Univ. Arizona Press, Tucson, AZ, in the press).
3. Luu, J. et al. *Nature* **387**, 573–575 (1997).
4. Duncan, M. J. & Levison, H. F. *Science* **276**, 1670–1672 (1997).
5. Edgeworth, K. E. *Mon. Not. R. Astron. Soc.* **109**, 600–609 (1949).
6. Kuiper, G. in *Astrophysics: A Topical Symposium* (ed. Hynek, J. A.) 357–424 (McGraw-Hill, New York, 1951).
7. Fernández, J. *Mon. Not. R. Astron. Soc.* **192**, 481–491 (1980).
8. Duncan, M., Quinn, T. & Tremaine, S. *Astrophys. J.* **328**, L69–L73 (1988).
9. Jewitt, D., Luu, J. & Chen, J. *Astron. J.* **112**, 1225–1238 (1996).
10. Malhotra, R. *Astron. J.* **110**, 420–429 (1995).
11. Levison, H. F., Stern, S. A. & Duncan, M. J. *Icarus* (submitted).
12. Morbidelli, A. & Valsecchi, G. B. *Icarus* (in the press).
13. Marsden, B. G. *Minor Planet Electronic Circulars* B19 (1997).
14. Fernández, J. A. & Ip, W.-H. *Icarus* **58**, 109–120 (1984).

Microbial genetics

The tinkerer's evolving tool-box

E. Richard Moxon and David S. Thaler

The neo-darwinian synthesis resulted from the realization that mendelian inheritance changed the darwinian model of biological evolution. Darwin assumed that parents' genes blend into each other in their offspring. This was problematic because a fitter variant's genes would blend upon mating, and become diluted into the gene pool. Mendelism solved this paradox by providing a mechanism for descent, whereby individual alleles remain intact and are not blended away through mating. This example shows that knowledge of the specifics of genetics — that is, the mechanisms by which variants are generated — sometimes has a profound effect on evolutionary theory.

Another example of this is now provided by two papers on pages 700 and 703 of this issue. In a theoretical study, Taddei *et al.*¹ predict that in asexual, clonal populations of *Escherichia coli*, the ability to generate mutator alleles (which can lead to an increase in mutation rate) increases the rate at which more fit individuals arise in the population. This occurs even if the mutator alleles remain at a very low frequency. Sniegowski and colleagues² have found that three of 12 independently propagated clonal populations of *E. coli*, which were serially cultured over 10,000 generations and thereby subjected to alternating periods of growth and stasis, had a

mutator phenotype which was due to defects in genes involved in DNA repair.

These papers transcend the assumption that mutations are spread more or less evenly through a population: this assumption was only reasonable when mutation was considered to be a direct result of random insults from outside an organism. Mutations are now known to be due to processing of the internal consequences of such damage, as well as to endogenous processes. The internal processing — a part of DNA metabolism — is carried out by gene products whose alleles have profound effects on the generation of variation. Alleles that predispose other genes to (possibly beneficial) mutations may hitchhike³, because the mutator allele and the phenotypically selected alleles of other genes are linked, especially in asexual clonal populations.

Taddei and colleagues¹ detailed population analysis, derived using the best numerical parameters available for *E. coli*, shows that mutators are a key aspect of adaptive evolution. The implications of their results are strengthened by a previous theoretical model⁴ showing that the effects of mutator alleles become larger as the evolutionary challenge is made more complex. The effect of incorporating mutators in the population increases exponentially as a function of the number of mutations required for a transition from one

local optimum in the evolutionary landscape to another, and how ill-adapted the intermediate genotypes are. There is an important difference between having the same number of mutations scattered randomly among individuals or, alternatively, having them clustered into a few highly mutating cells.

The inheritance of mutator alleles is not the only trick that bacteria have up their sleeve. Certain pathogens (viruses, bacteria and even large parasites) have subsets of genes that are excessively prone to mutation through, for example, slipped-strand mispairings, gene conversions and point mutations⁵. These hypermutable genes encode the surface molecules, such as adhesins or

invasins, that are involved in interactions with host molecules. Using just a few such loci, a population of microbes can use a combinatorial system (see box) to generate phenotypic variations which can influence many aspects of behaviour — antigenicity, motility, chemotaxis, attachment to host cells, resistance to desiccation, acquisition of nutrients and sensitivity to antibiotics. These hypermutable sequences have been called 'contingency loci' because they allow the pathogen to explore precipitous and unpredictable aspects of the host environment, while minimizing deleterious effects on fitness⁶. Duplications and recombination, both within and between genomes,

provide further options for combinatorial diversification⁷. Many of these diversity-generating mechanisms often act as a reversible binary switch.

In last week's issue, Bridges⁸ discussed the physiological modulation of mutation rate⁹, and alluded to possible relationships of such mechanisms with the debate on the fundamental and controversial issue of directed mutation¹⁰. Are microbes smart enough to put two-and-two together, and coordinate physiological modulation of mutation rate with focused action at contingency loci? Both of the new papers^{1,2} scrupulously avoid comment on directed mutation. It has been a tenet of molecular biology that genetic information is independent of events that occur outside, or even inside, the cell. The conventional view is that the environment selects among pre-existing variants — mutations arise without regard for their usefulness. But, intuitively, it seems advantageous for organisms to evolve mechanisms through which the environment could influence the genetic mechanisms that would alter the quantity and type of alleles in the repertoire on which selection acts.

The combination of contingency loci and physiological mutators goes some distance towards 'directed mutations', with no requirement for new molecular mechanisms or a reverse flow of information. The essentials of some of these ideas are touched upon in the 'hypermutable state' model proposed by Hall¹¹. Contingency loci provide a molecular mechanism by which a hypermutable state is localized to particular regions of the genome. Physiological modulation of mutation implies that cells of increased fitness leave the mutator state and resume growth. Inheritance and mutation of the alleles of DNA metabolism may accomplish almost the same tricks as the physiological modulation of mutation rate, albeit more slowly and by a different evolutionary mechanism.

Whether or not the mutation rate is an important variable in bacterial pathogenesis, the presence of mutators in natural populations of organisms¹² must be considered in the context of resistance to antibiotics¹³. An analogous situation may apply to infection with human immunodeficiency virus¹⁴, where a high mutation rate is probably a key factor in the pathogenesis of disease progression. For many infections, the use of combination therapies (that is, simultaneous treatment with several drugs) is a favoured therapeutic strategy. If mutations can give rise to resistance, the rare clones that become resistant to several antibiotics are likely to be mutators. Cancer is also a 'clonal pathogen' — mutator alleles are oncogenes. This was predicted two decades ago¹⁵, based on the reasoning that the odds of several coincident mutations leading to a cancer in the same cell are too small at the normal mutation rate.

The generation of variation is itself under genetic control, allowing a reflectivity — an

Algorithms for generating evolutionary variation

Pathogenic bacteria face particularly stringent tests of their adaptive potential because infections often occur within a short time frame (hours). During this crucial period, pathogens encounter the varying polymorphisms, immune repertoires and microenvironments of the host. In this race against time — with the escalation of host immune-clearance mechanisms and, perhaps, the administration of potent antimicrobials — the combinatorial effects of a minority subset of hypermutable (contingency) genes provides flexibility for adaptation.

Take the hypothetical genome of a pathogenic bacterium, comprising 2,000 genes including seven contingency genes (A–G), each of which can reversibly switch at a frequency of 10^{-3} per bacterium per generation. Assume that each gene is a binary genetic switch (for example, A to A'), with corresponding phenotypes a or a' up to g or g'. If these genes switch independently, this gives 128 different phenotypic possibilities. Suppose that a bacterium of phenotype a, b, c, d', e, f, g is optimal for colonization of a host's epithelial cells, but long-

term colonization is favoured by entry into host cells. Now suppose that bacteria with the phenotype a', b, c, d, e, f', g' (involving four switches from the original) are best able to invade and survive in the cells of the host: these bacteria will be selected as the adaptive phenotype. This is a rare event — about one in a trillion cells. But a population of cells with a 100-fold increase in mutation rate will, on average, contain an individual of the requisite genotype when the population size is only 10,000. This phenotypic variation, which is

stochastic with respect to the timing of switching but has a programmed genomic location, allows a large repertoire of phenotypic solutions to be explored, while minimizing deleterious effects on fitness.

The combinatorial strategy exploited by contingency loci is found elsewhere, generating the diversity seen by Mendel and in the influenza virus. Jacob¹⁹ spoke of "The mode of operation of the tinkerer... arranging various combinations to produce new objects of increasing complexity". The algorithm (tool-box) of the tinkerer also evolves:

The tinkerer's tool-box

Genetic determination of mutation rate by standard genes of DNA metabolism

- Polymerase editing
- Post-replication mismatch correction, mutS, mutL, mutH, mutU
- Chemical lesion processing

Physiological modulation of the generation of variation

- SOS
- Stringent response
- Carbon starvation
- Transient mutators
- Meiosis

Focusing change differentially in the genome

- Contingency loci
- Gyrase binding sites
- Duplication, deletion or inversion between repeats
- Site-specific recombination
- Hot and cold regions for generalized recombination

informational feedback — on the mechanisms by which diversity is generated in biological evolution^{4,16,17}. As E. G. Leigh¹⁸ wrote: “selection will hold mutators fully responsible, as it were, for both the good and the bad they cause”. The implications of this evolving feedback are of significance to biologists, mathematicians and others concerned with information flow in dynamical systems. □

E. Richard Moxon is at the University of Oxford Department of Paediatrics and Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK. David S. Thaler is at the Sackler Laboratory of Molecular Genetics and Informatics, The Rockefeller University, 1230 York Avenue, New York, New York 10021-6399, USA.

1. Taddei, F. *et al.* *Nature* **387**, 700–702 (1997).
2. Sniegowski, P. D., Gerrish, P. J. & Lenski, R. E. *Nature* **387**, 703–705 (1997).
3. Ruiz-Rubio, M. & Bridges, B. A. *Mol. Gen. Genet.* **208**, 542–548 (1987).

4. Magnasco, M. & Thaler, D. S. *Phys. Lett. A* **221**, 287–292 (1996).
5. Deitch, K. W., Moxon, E. R. & Welles, T. E. *Microbiol. Mol. Biol. Rev.* (in the press).
6. Moxon, E. R., Rainey, P. B., Nowak, M. A. & Lenski, R. E. *Curr. Biol.* **4**, 24–33 (1994).
7. Thaler, D. S. *Trends Ecol. Evol.* **9**, 108–110 (1994).
8. Bridges, B. A. *Nature* **387**, 557–558 (1997).
9. Wright, B. E. *FEBS Lett.* **402**, 4–8 (1997).
10. Cairns, J., Overbaugh, J. & Miller, S. *Nature* **335**, 142–145 (1988).
11. Hall, B. G. *Genetics* **126**, 5–16 (1990).
12. LeClerc, J. E., Li, B., Payne, W. L. & Cebula, T. A. *Science* **274**, 1208–1211 (1996).
13. Lipsitch, M. & Levin, B. R. *Antimicrob. Agents Chemother.* **41**, 363–373 (1997).
14. Leigh Brown, A. J. & Richman, D. D. *Nature Med.* **3**, 268–271 (1997).
15. Nowell, P. C. *Science* **194**, 23–28 (1976).
16. Thaler, D. S. & Messmer, B. T. in *Encyclopedia of Molecular Biology and Medicine* Vol. 2 (ed. Myers, R.) 407–414 (VCH, New York, 1996).
17. Thaler, D. S. *Science* **264**, 224–225 (1994).
18. Leigh, E. G. *Genetics* **73** (Suppl.) 1–8 (1973).
19. Jacob, F. *Science* **196**, 1161–1166 (1977).

Origins of life

The first two billion years

Eörs Szathmáry

The problems associated with defining the origins of, and transitions between, different levels of biological organization receive increased attention these days, and rightly so. New discoveries and models help us to account for the processes that resulted in new ways of storing and retrieving hereditary information. These processes often relied on the formation of higher-level evolutionary units from previously unlinked replicators — small units that can replicate independently¹. This was the subject of a recent meeting², organized by the Swedish Natural Science Research Council, at which topics ranging from the origin of the first replicators to the emergence of multicellular eukaryotes were discussed.

The origin of non-enzymatic replication is still an unsolved problem. Although artificial replicators can grow without a replication enzyme (replicase) in a test tube³, they are not a historically realistic recapitulation of what could have happened on the early Earth. But there are three promising lines of research: replication of nucleic acids on a surface; *in vitro* construction of a catalytic RNA (ribozyme), which could act as a generalized replicase; and formation of oligonucleotides by ligating together smaller nucleic-acid units.

Surface-bound template polymerization has been successful up to a length of 55 nucleotides³. At this length, ribozymic activity of an RNA sequence is also feasible. An RNA-dependent RNA polymerase ribozyme can copy templates of up to six nucleotides with remarkable efficiency⁴, although the ultimate aim would be to have a molecule that could copy any sequence, including

itself. Replicase ribozymes would be especially useful in studying autonomously evolving ‘ecosystems’ of molecules. For example, A. Eschenmoser (ETH, Zürich) asserts that pyranosyl-RNA (pRNA) is a possible substitute for the commonly occurring furanosyl variant (Fig. 1a): pRNA has a stronger and more selective base-pairing system than ordinary RNA. So why did nature first, maybe, choose pRNA, and why was it later abandoned?

Tetranucleotide-2',3'-cyclophosphates have also recently been shown to assemble

into oligomers of up to 36 nucleotides in dilute solution⁵ (Fig. 1b). This oligomerization is highly chiroselective — a homochiral template catalyses the formation of the correct phosphodiester junction between homochiral tetramers that have the same ‘handedness’ as the template.

The origin of biomolecular homochirality is another important aspect, because it is connected to the notion of replicators with limited or unlimited heredity^{1,6}. For replicators that have a limited ability to pass on characteristics, the number of individuals will typically exceed the number of possible sequences — an example is a replicating oligonucleotide. Contemporary nucleic-acid-based replicators in living systems have, in contrast, unlimited heredity, and a museum showing all of the possible sequences up to a certain length would be larger than the Universe.

Imagine that one starts with a mixture of homochiral templates of D- and L-enantiomers, and the degree of oligomerization grows with time: each chiral template gives rise to its own kind. Eventually, the supply of raw materials will be exhausted, and it will no longer be possible to have at least one copy of each sequence. Some sequences will be present in only one enantiomeric form or the other, so the symmetry of the molecular population will be spontaneously broken, paving the way for the passage to unlimited heredity. One population will now become more successful than the other, because it will consist of fitter sequences; and, ultimately, the population with the opposite sense of chirality will be competitively displaced⁵.

But what about the origin of homochiral tetramers, or enantiomeric excesses in gen-

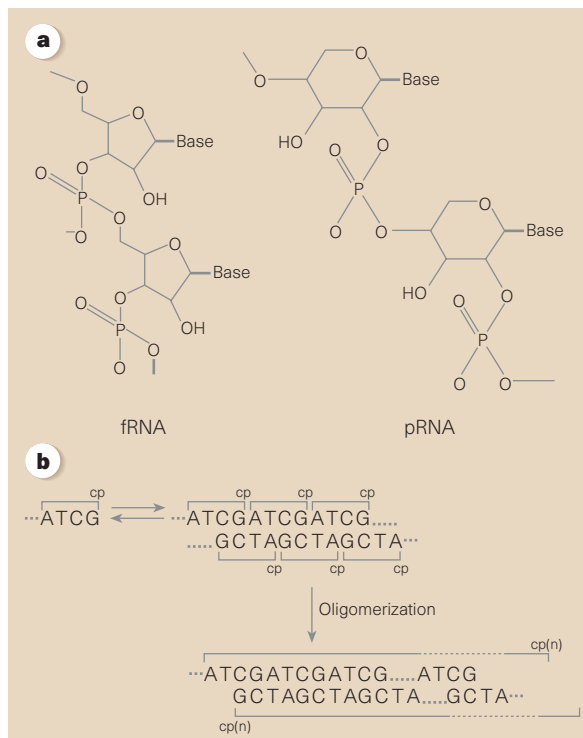


Figure 1 The origins of nucleic-acid-like molecules. a, The common (furanosyl) form of RNA (fRNA) may have superseded the pyranosyl form (pRNA), in spite of the fact that pRNA has a stronger and more selective base-pairing system. b, In dilute solution, tetranucleotide cyclophosphates (cp) can assemble into oligomers up to 36 nucleotides in length. The oligomerization is chiroselective, so only tetramers that have the same chirality as the template are joined — when one of the (D)-ribopyranosyl units at any position of a homochiral (D)-tetramer is replaced by a corresponding (L)-unit, the rate of oligomerization is reduced by about two orders of magnitude.

* Scientific Forum on the Origin of Life, Arken, Gothenburg, Sweden, 7–9 April 1997.

eral? J. Mayo Greenberg (Huygens Laboratorium, Leiden) emphasized the importance of the organic molecules that were delivered to the early Earth by comets and meteorites. Remarkably, circularly polarized light from neutron stars can probably yield, in interstellar dust, enantiomeric excesses of ten per cent or even higher — this agrees with the degree of chirality of amino acids in the Murchison meteorite⁷.

Although nucleic-acid-like molecules receive a lot of attention, G. Wächtershäuser (Munich) argues that the first replicators with limited heredity were much smaller molecules, with analogue rather than digital replication. Whereas analogue replication proceeds piecemeal, digital replication is a modular process. It was recently found that an archaic, reductive citric-acid cycle could have been primed in an 'iron-sulphur' world — co-precipitated NiS and FeS can convert CO and CH₃SH into an activated thioester (CH₃-CO-SCH₃) at hydrothermal temperatures (100 °C)⁸. The next, eagerly awaited step is the experimental demonstration of a functioning cycle.

So could life have originated at high temperatures? Phylogenetic analyses indicate that the last common prokaryote ancestor was a hyperthermophilic organism⁹, presumably growing chemolithoautotrophically on CO₂ (K. O. Stetter, Univ. Regensburg). But it is not yet clear whether hydrogen-driven ecosystems in deep granitic rock aquifers¹⁰ are relevant to the origin of life. In fact, it is frustrating that the extremely poor rock and fossil records of the Archaean (3.8–2.5 billion years ago) do not tell us anything about the nature of the primordial atmosphere, impact frustration or the existence of an RNA world (J. W. Schopf, CSEOL Geological Building, Los Angeles).

How far the analogue replicators could have evolved before the advent of digital replicators is an open problem^{1,6}, both experimentally and theoretically. The lambda and other calculi (which can handle the operations of functions on other functions in the most general way) have been applied to reflect some basic principles of chemistry and, although there were serious shortcomings in this approach⁶ a few years ago (for example, with interchangeability of reactants and conservation of matter), they have partly been eliminated. The potential now lies in modelling chemical organization and evolution *in abstracto*¹¹.

The last common prokaryotic ancestor had ribosomes, so it must have translated proteins using a genetic code. It would have been advantageous to introduce amino acids into an RNA world (as coenzymes for ribozymes), and 'coding' oligonucleotide handles, linked to the amino acids, could have helped the ribozymes to recognize them — reminiscent of present-day aminoacyl-

tRNA¹. (Note that various 5-substituted uracils can arise under prebiotic conditions¹².) So the genetic code may have been a pre-adaptation for translation, and this idea is experimentally testable.

The origin of eukaryotes was a later development, the most controversial aspect of which is the origin of microtubules and cilia by symbiosis with spirochaetes (L. Margulis, Univ. Massachusetts). Ultimately, the gene sequences of ciliary proteins should be decisive; the location of DNA (whether in the basal bodies or in the nucleus) is of secondary importance. J. T. Bonner (Princeton Univ.) called attention to a conspicuous pattern in the many independent origins of (prokaryotic and eukaryotic) multicellularity — aquatic forms arise by the division and sticking-together of cells, whereas terrestrial forms arise through aggregation (with the exception of the actinomycetes).

I expect that considerable experimental and theoretical advances will now be made in studies of autonomous replication; synthesis of biologically important chemical supersystems; and the transition from RNA to the nucleic acid and protein worlds; and that there will be a deeper understanding of the origin of multicellular eukaryotes. □

Eörs Szathmáry is in the Department of Plant Taxonomy and Ecology, Eötvös University, Ludovika tér 2, H-1083 Budapest, and at the Collegium Budapest, Szentháromság u. 2, H-1014 Budapest, Hungary.

1. Maynard Smith, J. & Szathmáry, E. *The Major Transitions in Evolution* (Freeman, Oxford, 1995).
2. von Kiedrowski, G. *Angew. Chem. Int. Edn Engl.* **25**, 932–935 (1986).
3. Ferris, J. P., Hill, A. R., Liu, R. & Orgel, L. E. *Nature* **381**, 59–61 (1996).
4. Eklund, E. H. & Bartel, D. P. *Nature* **382**, 373–376 (1996).
5. Bolli, M., Micura, R. & Eschenmoser, A. *Chem. Biol.* (in the press).
6. Szathmáry, E. *Proc. R. Soc. Lond. B* **260**, 279–286 (1995).
7. Cronin, J. R. & Pizzarello, S. *Science* **275**, 951–955 (1997).
8. Huber, C. & Wächtershäuser, G. *Science* **276**, 245–247 (1997).
9. Bock, G. R. & Goode, J. A. (eds) *Evolution of Hydrothermal Ecosystems on Earth (and Mars?)* (Wiley, Chichester, 1996).
10. Pedersen, K. *Earth Sci. Rev.* **34**, 243–260 (1993).
11. Fontana, W. & Buss, L. W. in *Boundaries and Barriers* (eds Casti, J. & Karlquist, A.) 56–116 (Addison-Wesley, Reading, MA, 1996).
12. Roberston, M. P. & Miller, S. L. *Science* **286**, 702–705 (1995).

Corrections

● In the obituary of Edward M. Purcell by Nicolaas Bloembergen (*Nature* **386**, 662; 1997), Purcell's lecture on the locomotion of bacteria in fluids was cited as *Life at High Reynolds Number*, rather than correctly as *Life at Low Reynolds Number*.

● The figure in David Jablonski's News and Views article ("Progress at the K-T boundary": *Nature* **387**, 354–355; 1997) was misattributed. It was modified, not from reference 17, but from ref. 15 (Marshall, C. R. in *The Adequacy of the Fossil Record* (eds Donovan, S. K. & Paul, C. R. C.) (Wiley, Chichester, in the press). Thanks are due to Charles Marshall for helping to prepare the modified version of the figure.

Daedalus

Pure liquid sound

In the hi-fi world, solid-state electronics is in retreat. Valves (vacuum tubes) are displacing transistors and integrated circuits. More curious still, strange new passive components are being recommended — silver, titanium or carbon wires and cables, gold foil capacitors, ultra-linear potentiometers, and so on. Suspiciously, all these components are vastly expensive. But, says Daedalus, they may have some basis in physics. One claim is that ordinary copper cable has rectifying tendencies, as exploited in the old copper oxide rectifier. Indeed, any soldered joint or impure metal may rectify, or generate thermoelectric voltages, or fail to obey Ohm's law. Test instruments may not be able to detect these tiny imperfections, but the ears of dedicated enthusiasts can.

So Daedalus is abandoning the solid state. He recalls that the nineteenth century standard of resistance was a column of liquid mercury. Being isotropic, it had no rectifying properties. In the 1960s, a sodium cable sheathed in polythene was tested for power transmission. Overloading it melted the sodium in its sheath, when the rise in resistance limited the current.

DREADCO's new liquid-state amplifier takes this technology to its logical conclusion. Its conductors are sodium-potassium alloy, which is liquid at room temperature (mercury would never pass the safety regulations). The whole circuit, including the transformer windings which get power into the system and the speaker coils which take it out again, uses polythene tubes filled with the alloy. Resistors and potentiometers are produced by squeezing the tubing with a clip to reduce its cross-section. Capacitors are twin narrow chambers of the alloy separated by a very thin polythene septum. No soldered joints or dissimilar junctions are needed at all.

The amplifying components will be based on the old mercury-arc rectifier, with its liquid electrodes. DREADCO physicists are inventing sodium-potassium alloy arc rectifiers and valves. Sadly, the valves need at least one solid-state component, a wire grid; but Daedalus hopes that a sufficiently expensive metal, platinum say, will prove adequate.

The liquid-state amplifier will transform hi-fi. It will sweep away all solid-state imperfections in a torrent of flawless sound. Its cost will be as chilling and its heat as warming as that of any valve amplifier. The sodium glare from its arc rectifiers will even light the room.

David Jones

Obituary

John C. Eccles (1903–97)

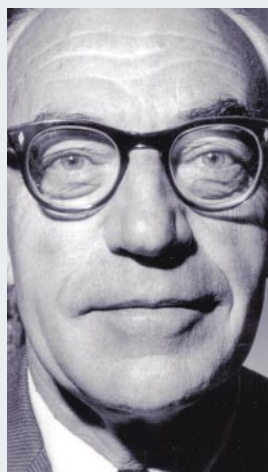
Neuroscientist who discovered synaptic inhibition

On 2 May 1997, the world's academic community lost John C. Eccles — a great hero of neuroscience. Among his many accomplishments, he discovered synaptic inhibition between nerve cells for which, with Allen Hodgkin and Andrew Huxley, he was awarded the 1963 Nobel Prize for Physiology or Medicine. As well as pioneering modern research on the physiology of nerve cells and synapses, he was a great teacher whose enthusiasm attracted many young researchers to his laboratories. He encouraged many others through his books and talks, and he was also a philosopher who enjoyed trying to solve difficult brain–mind problems.

Eccles was born in 1903 in Australia. After graduating from Melbourne University Medical School, he studied in Oxford under Charles Sherrington, from whom he learned a great deal about the classic concepts of reflexes, such as the convergence and divergence of synaptic connections, excitatory and inhibitory states in a pool of motor neurons (nerve cells innervating muscles), and the integrative action of the central nervous system. Clearly, Sherrington was a strong role model, although Eccles' life at Oxford was enriched by interactions with his contemporaries, including Ragnar Granit and J. Z. Young.

In 1937, John Eccles returned to Australia, where he was appointed director of the Kanematsu Memorial Institute of Pathology, and was joined by Bernard Katz and Stephen Kuffler. At that time there was worldwide debate as to whether synaptic transmission is electrical or chemical. Eccles championed the electrical-transmission hypothesis, proposing that impulses travel across synapses by means of action currents, just as they travel along a nerve fibre. He even speculated that inhibition is induced by reverse-flow of action currents through the postsynaptic membrane. These were early working hypotheses which he later tested experimentally, leading to his great discovery.

Eccles became a professor of physiology at the University of Otago Medical School, New Zealand, in 1944. There, along with L. G. Brock and Jack Coombs, he discovered synaptic inhibition. When he stimulated a nerve — and so induced an excitatory state in a motor neuron pool — he detected



EPSPs — that is, inhibitory postsynaptic potentials. These potentials, occurring with a small but distinct delay after nerve impulses, were inconsistent with the electrical-transmission hypothesis, because they showed that synaptic transmission must involve a chemical process that mediates either excitation or inhibition.

In 1951, Eccles moved to Canberra, which subsequently became a Mecca for neurophysiologists. There, he experienced his most productive 15 years, with many colleagues from all over the world, including his daughter, Rose Eccles. From my stay in Canberra, between 1959 and 1962, I have a hundred stories to tell about this Golden Age of Eccles' research. He performed experiments twice a week, starting each experiment at 8.30 a.m., and continuing until 3 a.m. the next morning. He then drove home, giving rides to his colleagues if they had no cars. Every Friday evening, a seminar was held on the work done in his department — he was not happy if someone merely introduced other people's work. And with the approach of the weekend, the atmosphere in the department became tense: we were exhausted from the hectic work and vigorous discussions, and our moods tended to deteriorate. But each Monday morning, 'The Prof.' reappeared in the lab, marvellously refreshed. I believe that the secret to his rejuvenation was the time that he spent in his big garden and in simple study at home.

Near the end of his time in Canberra, Eccles extended his work from the spinal cord to higher levels of the central nervous system, including the hippocampus, thalamus and cerebellum. In 1966, he moved to the newly launched Institute for Biomedical Research of the American Medical Association in Chicago, where he

excitatory postsynaptic potentials (EPSPs) in individual motor neurons. Surprisingly, when he induced an inhibitory state in the motor-neuron pool, he detected mirror images of

continued to work on the cerebellum. He prophesied that "since we now know the blueprint of the cerebellum, its functional principles will soon be discovered". But his time in Chicago was short and, in 1968, he moved to the State University of New York at Buffalo. There he set the final target of his academic career — the solution of brain–mind problems — and he pursued this, even after his retirement in 1975, when he moved to Switzerland to live out the rest of his years with his wife Helena.

It will probably take some time before Eccles' contributions to the field of solving brain–mind problems can be properly evaluated. To me, there are three main points at issue. First, his proposal that synaptic transmission includes a quantum-mechanical process in a probabilistic field seems to represent a feeling in his bones that we are still lacking something essential in our knowledge of nerve cells and synapses. Second, he proposed that the supplementary motor cortex acts as a liaison through which the mind interacts with the brain. Experimental evidence indicates that a will to perform movements emerges from the supplementary cortex, yet most neuroscientists believe that neuronal activity in this cortex is driven by neuronal activity in other parts of the brain and not from outside it. However, nobody knows how, in fact, it is driven. Finally, he warned that there are currently no clear perspectives towards the solution of brain–mind problems. We anticipate that new discoveries will follow from our approaches to working out the mechanisms of higher-order brain functions, such as learning and memory, perception, movement control, emotion, thought, language and attention. But in terms of the questions of how our subjectivity emerges and where it is represented in our brains, there is still a large gap in our knowledge that we do not know how to bridge.

On a day 34 years ago — when he visited Japan for the first time — Eccles impressed me with the words that he was irritated whenever he could not understand something. Although he has now passed away, his great desire to understand everything, including our subjectivity, has been transferred to the minds of many of his successors. Sir John Eccles was a great scientist and a great man, whose experiences spanned almost the entire twentieth century, and who will be remembered with great respect for ever.

Masao Ito

Masao Ito is in the Laboratory for Memory and Learning, Frontier Research Program, RIKEN, Wako, Saitama 351-01, Japan.

Tracking fish with electronic tags

The distributions of many species of fish show pronounced seasonal changes as a result of migration¹. We are using long-term electronic tagging to study the migratory behaviour of fish in the North Sea. Using simple measurements of depth and temperature, we have found that we can reconstruct the tracks of plaice (*Pleuronectes platessa*) migrating between the southern North Sea and their spawning areas in the eastern English Channel or northeast coast of England. In this way we show that fish can visit more than one spawning area within a single spawning season and that

rates of movement are often ten times faster than those deduced from conventional mark-recapture experiments.

In the North Sea, plaice, cod and several other species migrate by selective tidal stream transport²⁻⁶. Fish leave the sea bed at or shortly after slack water, swim up into mid-water and are carried downstream by the tide before returning to the bottom about six hours later. Maturing fish use one tidal stream to reach their spawning grounds; fish that have spawned use the opposing stream to return to their feeding grounds⁷.

Until recently we have only been able to

track individual fish for a few days at a time using a research vessel, sonar and acoustic tags. Now our engineers have developed an electronic tag that records depth (0–100 m $\pm$ 0.2 m) and water temperature (-4 to 23 $^{\circ}\text{C} \pm 0.03$ $^{\circ}\text{C}$), allowing us to observe over long periods and replicate our observations. The Mk 1 tag, which was specifically designed for flat-fish, stores 32,000 data samples and weighs about 55 g in air (23 g in sea water). The Mk 3 tag, which can be used on roundfish as well as flat-fish, is smaller (16 g in air), can store over 500,000 data samples and also records light intensity.

We attached 303 Mk 1 tags to large (over 40 cm long) maturing female plaice in the southern North Sea between December 1993 and February 1997. Thirty-seven tags have so far been returned through the commercial fishery, producing 1,946 days of data. Nine tags recorded continuously for over 100 days and 3 for over 200 days; one tag was returned after 300 days at sea. Several fish spawned whilst at liberty, and it seems that large plaice can tolerate the Mk 1 tags for long periods without migration or spawning being seriously inhibited.

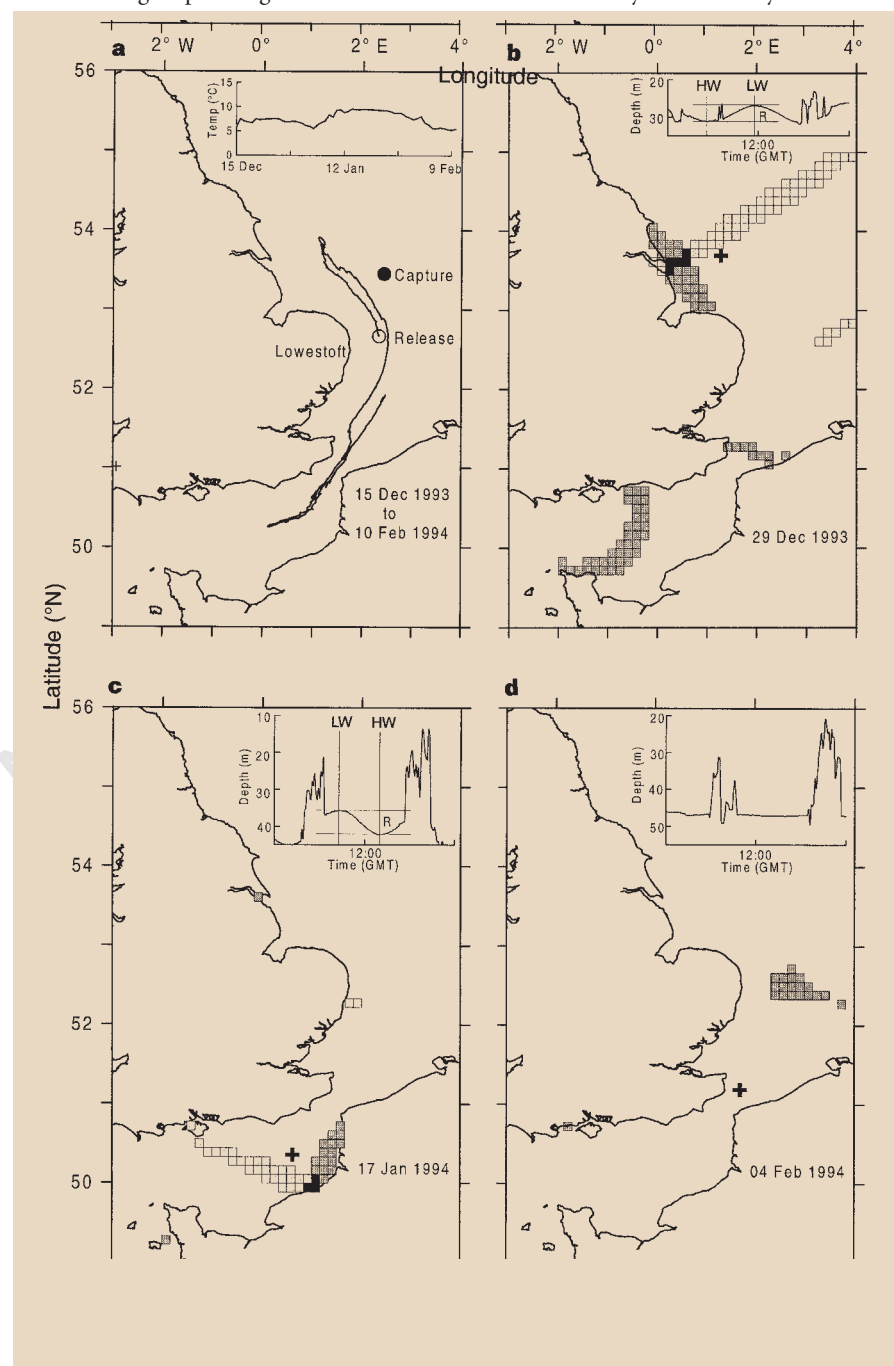


Figure 1 a, The reconstructed track for a maturing female plaice (48 cm long) tagged with a Mk 1 data storage tag (DST 08). **b–d**, Independent confirmation of geographical location at 3 positions based on measurements of tidal range (R) and times of high water (HW) recorded by the tag (insets). Independent estimates of location (crosses) were obtained with the Proudman Oceanographic Laboratory's numerical storm-surge model¹², which calculates tidal data on a 12-km grid. This model was used to identify areas of similar (± 0.4 m) tidal range (dark stipple) and similar (± 10 minutes) times of HW (light stipple) to those recorded by the tag (congruent areas are indicated in black). In the North Sea, the tidal wave rotates around three amphidromes (points of no tide where $R=0$). Geographical location is obtained from bi-coordinate grids formed by co-tidal lines (linking points with the same tidal phase) radiating from the amphidromes and co-range lines (linking points of equal tidal range) centred on the amphidromes. The absence of a sinusoidal tidal wave (inset in **d**) shows that the fish was close to the amphidrome at $52^{\circ} 30' \text{ N}$, $03^{\circ} 03' \text{ E}$ on 4 February. Comparison of the daily record of seawater temperature with synoptic charts of sea surface temperature (Bundesamt für Seeschifffahrt und Hydrographie, Hamburg) provides a further check on geographical location. The temperature record (inset in **a**) shows that the fish spent 12 days in water warmer than 9°C , a temperature observed in the eastern Channel, but not the North Sea during January and February 1994.

The track of one plaice, caught on 10 February 1994 after 56 days, demonstrates the information we can obtain with the new tags. Although release and recovery positions were only 88 km apart, the reconstructed track (Fig. 1a) shows that the fish travelled more than 900 km, first moving north to the Flamborough Off Ground and then south to the eastern English Channel — both important spawning grounds for plaice — before being caught by a Dutch trawler in the southern North Sea. This result was most unexpected, as it is usually assumed that plaice are faithful to one spawning ground, at least within a single spawning season. The track reconstruction⁸ assumes that the fish migrated by selective tidal stream transport², and that, when off the bottom, swam downstream at an average speed of 0.6 body lengths per second⁹, while being carried over the ground by the tide. The track is confirmed by independent estimates of geographical position obtained from depth measurements made when the fish was stationary on the sea bed for one or more tidal cycles (Fig. 1b–d), and by a comparison of local sea temperature with recordings from the tag (Fig. 1a).

Nine of the 17 tracks we have reconstructed so far show substantial migrations south into the eastern English Channel or north along the east coast of England. Three tracks show two or more reversals similar to those described above, and the temperature and hydrostatic data again provide independent confirmation of the scale of movement. The tracks show that individual fish can move rapidly over large distances and suggest that migration rates (over 20 km per day where tidal streams are fast) can often be ten times faster than those deduced from mark-recapture experiments¹⁰.

Our findings illustrate how observations of individual fish can shed new light on the behaviour of exploited populations. The results are relevant to questions of stock identity and the definition of biologically realistic quota areas; they also provide a basis for assessing the impact of technical conservation measures. Closed areas, which are already used to underpin quota management within the European common fisheries policy¹⁰, have recently been suggested as a method of managing cod in the northwest Atlantic following the catastrophic collapse of the Newfoundland fishery¹¹. In addition to practical applications, the data provide new insights into the environmental cues used by migratory fish and the sensory systems underlying their sophisticated patterns of behaviour.

J. D. Metcalfe, G. P. Arnold

Centre for Environment, Fisheries and Aquaculture Science,

Lowestoft Laboratory, Lowestoft, Suffolk NR33 0HT, UK

e-mail: j.d.metcalfe@cefas.co.uk

1. Harden Jones, F. R. *Fish Migration* (Arnold, London, 1968).
2. Greer Walker, M., Harden Jones, F. R. & Arnold, G. P. *J. Cons. Int. Explor. Mer* **38**, 58–86 (1978).
3. de Veen, J. F. *Neth. J. Sea Res.* **12**, 115–147 (1978).
4. Arnold, G. P. in *Animal Migration* (ed. Aidley, J. D.) 55–79 (Cambridge Univ. Press, 1981).
5. Rijnsdorp, A. D., von Stralen, M. & van Veer, H. W. *Trans. Am. Fish. Soc.* **114**, 461–470 (1985).
6. Arnold, G. P., Greer Walker, M., Emerson, L. S. & Holford, B. H. *ICES J. Mar. Sci.* **51**, 207–232 (1994).
7. Arnold, G. P. & Metcalfe, J. D. *Mar. Biol.* **127**, 151–160 (1996).
8. Arnold, G. P. & Holford, B. H. *ICES J. Mar. Sci.* **52**, 981–990 (1995).
9. Metcalfe, J. D., Arnold, G. P. & Webb, P. W. *J. Mar. Biol. Assoc. UK* **70**, 149–162 (1990).
10. Rijnsdorp, A. D. & Pastoors, M. A. *ICES J. Mar. Sci.* **52**, 963–980 (1995).
11. Walters, C. & Maguire, J.-J. *Rev. Fish Biol. Fish.* **6**, 125–137 (1996).
12. Flather, R., Proctor, R. & Wolf, J. in *Computer Modelling in the Environmental Sciences* (eds Farmer, D. G. & Rycroft, M. J.) 15–30 (Clarendon, Oxford, 1991).

Long-term potentiation in awake mutant mice

The relationship between long-term potentiation (LTP) and spatial learning has been explored in a variety of genetically engineered mice with deletions of specific genes¹. With few exceptions, LTP in these animals has been studied in the hippocampal slice preparation. The conditions required to elicit LTP *in vitro*, however, may not be comparable to those in the intact animal.

LTP is readily induced in the dentate gyrus of the anaesthetized or awake rat, but its induction *in vitro* requires the addition of a GABA_A antagonist such as bicuculline to the bathing medium². In mice lacking the cell-adhesion molecule *Thy-1*, we were unable to elicit LTP in the dentate gyrus of the anaesthetized mouse, whereas *in vitro*, with inhibition by GABA suppressed, the magnitude of LTP was the same in

Thy-1^{−/−} and wild-type littermates³. By adding bicuculline to the solution in the recording pipette, however, we were able to 'rescue' LTP in the dentate gyrus of the anaesthetized *Thy-1*^{−/−} mouse.

These observations raise the question whether LTP can be induced in the dentate gyrus of the awake, freely moving *Thy-1*^{−/−} mouse. *Thy-1*^{−/−} mice show no deficit in performance in the Morris water maze, and our previous results in the anaesthetized animal suggest that LTP in the dentate gyrus may not be necessary for spatial learning. We have developed techniques to monitor LTP in the awake mouse⁴, and have now examined ten *Thy-1*^{−/−} mice and five control mice (two wild-type and three heterozygotes) from the same breeding colony as the earlier study³.

Under pentobarbitone anaesthesia, we placed a stimulating electrode in the angular bundle to activate perforant path fibres projecting to granule cells, and a recording electrode in the hilus of the dentate gyrus to monitor evoked field responses (see ref. 4 for details). Evoked responses were recorded through a miniaturized headstage.

Animals were allowed to recover for a week. During the following week they were handled daily, and familiarized with the recording box. In the third week, evoked potentials were sampled daily. After stable responses had been recorded for two days, tetanic stimulation was applied, and the effect on the evoked response monitored over the next three days. At the end of the testing period, tail clips were taken for genetic typing³.

Although the average magnitude of LTP in the awake *Thy-1* knockouts is significantly lower than in control animals (Fig. 1a), unambiguous LTP was nevertheless observed in 5 of 10 knockout animals (Fig. 1b). This contrasts with the situation in the anaesthetized animal, where we observed

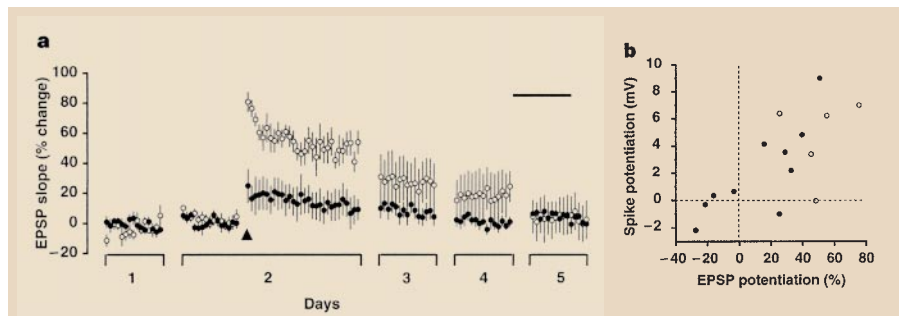


Figure 1 a, Slope of the field EPSP evoked in the dentate gyrus in *Thy-1*^{−/−} mice ($n=10$, filled circles), and in control littermates ($n=5$, open circles). Baseline responses evoked by test stimuli at 0.033 Hz were measured on 2 days, after which a tetanus (6 series of 6 trains of 6 stimuli at 400 Hz; 100 ms between trains, 20 s between series) was delivered (arrow). Responses to the test intensity were measured for 60 min after LTP induction, and for 30 min each on the next three days. Each point represents the mean of 4 consecutive responses normalized to the mean baseline value. Bar represents 30 min. The magnitude of LTP in *Thy-1*^{−/−} mice was significantly less than in controls only on the day of induction ($P<0.05$, ANOVA). **b**, LTP in the dentate gyrus of individual *Thy-1*^{−/−} (closed circles) and control (open circles) mice, plotted as mean absolute change in population spike amplitude, measured 30–60 min after tetanus, against the corresponding percentage change in EPSP slope.

no LTP in any of 13 animals³. These results weaken the evidence for our earlier conclusion that LTP in the dentate gyrus may not be necessary for spatial learning. The reduction in LTP in the awake *Thy1*^{-/-} animal may simply be insufficient to produce a spatial learning impairment.

Neither the *in vitro* preparation nor the anaesthetized mouse necessarily provides an accurate prediction of the potential for LTP in the awake animal. The wide variability in the degree of LTP exhibited by animals in the same alert, awake state (a variability which may reflect individual differences in the level of inhibitory tone) prompts the cautionary observation that attempts to correlate LTP with behaviour are unlikely to be interpretable unless both are assessed in the same animal.

M. L. Errington, T. V. P. Bliss

Division of Neurophysiology,
National Institute for Medical Research,
London NW7 1AA, UK
e-mail: merrington@nimr.mrc.ac.uk

R. J. Morris

Department of Experimental Pathology,
UMDS Guy's Hospital, London SE1 9RT, UK

S. Laroche, S. Davis

Neurobiologie de l'Apprentissage et de la Mémoire,
CNRS URA 1491, Université de Paris Sud,
91405 Orsay, France

1. Grant, S. G. N. *Curr. Opin. Neurobiol.* **4**, 687–692 (1994).
2. Wigström, H. & Gustafsson, B. *Nature* **301**, 603–604 (1983).
3. Nosten-Bertrand, M. *et al. Nature* **379**, 826–829 (1996).
4. Davis, S. *et al. J. Neurosci. Meth.* **75**, 75–80 (1997).

Rhodopsin evolution in the dark

The gene for the visual pigment rhodopsin has been extensively studied from biochemical, molecular and evolutionary perspectives. This makes it ideal for investigating the relationship between protein structure and function^{1,2}, and the effects of loss of functional constraint on the evolution of a gene. We specifically addressed the question of what happens when there is no light available by studying rhodopsin genes from three pairs of cave-dwelling and surface-dwelling freshwater crayfish species. Contrary to predictions, we found no differences in the rate of evolution between the cave and surface species or between the conserved and variable structural motifs of the rhodopsin protein. This suggests that rhodopsin might have a previously unknown function in the absence of light.

We sequenced a 900-base-pair segment of the rhodopsin gene from three species pairs of crayfish, each from a different genus (*Cambarus*, *Orconectes* and *Procambarus*), as well as the gene from an outgroup species, *Cambarellus schufeldtii*. Each species pair consisted of a cave-dwelling

Table 1 Synonymous and non-synonymous nucleotide substitutions

	Synonymous		Non-synonymous		P value
<i>C. hubrichti</i> C	13	(7.30–22.3)	6	(2.61–13.3)	0.206
<i>C. maculatus</i>	7	(3.29–14.3)	6	(2.61–13.3)	
<i>O. australis</i> C	3	(0.82–8.81)	10	(5.32–18.3)	0.127
<i>O. virilis</i>	8	(3.77–15.8)	9	(4.46–17.3)	
<i>P. orcinus</i> C	2	(0.36–7.29)	2	(0.36–7.29)	0.255
<i>P. seminolae</i>	6	(2.61–13.3)	1	(0.052–5.76)	
Total Cave	18	(11.2–28.3)	18	(11.2–28.3)	0.157
Total Surface	21	(13.3–32.3)	16	(9.60–25.9)	

A comparison between the number of synonymous and non-synonymous nucleotide substitutions in the cave (C) and surface lineages. Numbers in parentheses are the 95% confidence limits based on sampling from a Poisson distribution¹². P values were determined using Fisher's Exact Test for independence.

species and its closest surface-dwelling relative. Using phylogenetic information about the species relationships^{3–5}, it is possible to reconstruct nucleotide substitutions and amino-acid replacements along the two independent lineages of each pair, and test the effects of loss of functional constraint on the rhodopsin gene (Fig. 1).

Our expectation was that, with a loss of functional constraint, there would be a significant difference in nucleotide substitutions between cave and surface lineages, with the cave lineages having a higher rate of evolution, similar to that in pseudogenes⁶. In addition, we expected that the amino-acid replacements would be distributed randomly over the structure of the protein, irrespective of known functional constraints.

Ancestral states for both nucleotide and amino-acid changes were reconstructed using the maximum-likelihood procedure⁷

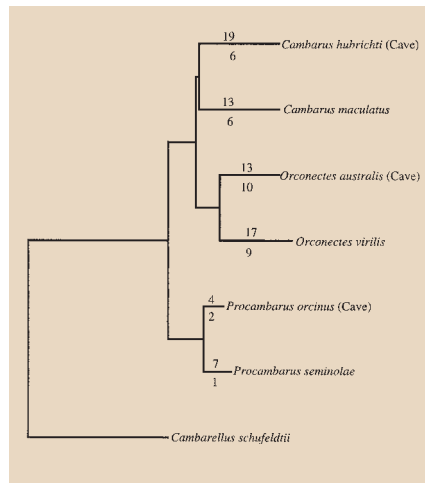


Figure 1 Maximum-likelihood reconstruction of nucleotide substitutions and amino-acid replacements for the crayfish phylogeny. Phylogenetic relationships were reconstructed with morphological^{3,4} and nucleotide sequence data⁵, independent of rhodopsin data. The number of nucleotide substitutions (above the branch) and amino-acid replacements (below the branch) was estimated using the programs BASEML and CODEML in the PAML package⁸. Branch lengths are proportional to the amount of change. Similar results were obtained using MacClade⁹.

of PAML⁸ software. Although there was a large number of changes along the phylogeny, the numbers of nucleotide substitutions along the cave and surface lineages were nearly identical (Fig. 1). Using a sign test, we could not reject the null hypothesis that there was no difference in the number of substitutions in all three lineages ($P=0.452$). Amino-acid replacements were also nearly equal in number (Fig. 1; $P=0.367$).

Next we tested to see if habitat (cave or surface) was independent of substitution pattern. We determined whether nucleotide substitutions were synonymous (substitutions that do not result in amino-acid replacement) or non-synonymous (substitutions that do result in amino-acid replacement) using the maximum-likelihood reconstructions. We then compared these values to habitat type, and again failed to reject the null hypothesis of independence (Table 1). This indicates that substitution pattern was not affected by surface/subterranean environmental differences. Indeed, in comparing nucleotide substitution matrices we found no significant difference between the cave lineages and the surface lineages ($P=0.2558$, Kolmogorov–Smirnov test). So there is no detectable difference in selection pressures on the patterns of nucleotide substitutions.

Finally, we examined amino-acid replacements related to different structural motifs of the rhodopsin protein. We partitioned the protein into structural units and identified the number of amino-acid replacements in these units. Again, we found no significant differences between the cave and surface lineages in the distribution of amino-acid replacements with respect to the secondary protein structure ($P=0.1549$, Kolmogorov–Smirnov test).

These lines of evidence indicate that there has been no loss of functional constraint for rhodopsin in the cave lineages of crayfishes, which in turn suggests that the protein is still functional, contrary to previous studies of cave-adapted organisms^{6,9}. Rhodopsin cannot have its conventional function, as there is no light available to initiate a biochemical cascade. We conclude

that rhodopsin has an additional, previously unrecognized function, perhaps a role in circadian rhythms^{10,11}, unrelated to light absorption.

Keith A. Crandall

Department of Zoology and M. L. Bean Museum,
Brigham Young University,
Provo, Utah 84602-5255, USA
email: keith_crandall@byu.edu

David M. Hillis

Department of Zoology
and Institute of Cellular and Molecular Biology,
University of Texas, Austin,
Texas 78712-1064, USA

1. Yokoyama, S. & Yokoyama, R. *Ann. Rev. Ecol. Syst.* **27**, 543–567 (1996).
2. Yokoyama, S. *Mol. Biol. Evol.* **12**, 53–61 (1995).
3. Hobbs, H. H. Jr *Smithson. Contr. Zool.* **164**, 1–32 (1974).
4. Hobbs, H. H. Jr in *Freshwater Crayfish: Biology, Management and Exploitation* (eds Holdich, D. M. & Lowery, R. S.) 52–82 (Timber, Portland, 1988).
5. Crandall, K. A. & Fitzpatrick, J. F. Jr. *Syst. Biol.* **45**, 1–26 (1996).
6. Yokoyama, S., Meany, A., Wilkens, H. & Yokoyama, R. *Mol. Biol. Evol.* **12**, 527–532 (1995).
7. Yang, Z., Kumar, S. & Nei, M. *Genetics* **141**, 1641–1650 (1995).
8. Yang, Z. *PAML, Phylogenetic Analysis by Maximum Likelihood* (Inst. Mol. Evol. Genet., Pennsylvania State Univ., 1995).
9. Hendriks, W., Leunissen, J., Nevo, E., Bloemendal, H. & de Jong, W. W. *Proc. Natl Acad. Sci. USA* **84**, 5320–5324 (1987).
10. Argamaso, S. M. *et al. Biophys. Chem.* **56**, 3–11 (1995).
11. Sassone-Corsi, P. *Cell* **78**, 361–364 (1994).
12. Crow, E. L. & Gardner, R. S. *Biometrika* **46**, 441–453 (1959).
13. Maddison, W. P. & Maddison, D. R. *MacClade: Analysis of Phylogeny and Character Evolution* (Sinauer, Sunderland, MA, 1992).

Chirality errors in nucleic acid structures

The prevalence of errors as opposed to true anomalies in protein structures was discussed in Correspondence last year¹. We have analysed the nucleic acid structures in the Brookhaven Protein Data Bank using a simple automatic procedure (program available at <http://www.mbi.ucla.edu/people/peter/chiral.html>) to determine the configurations at tetrahedral chiral centres. It appears that a number of structures containing significant “chirality errors that no-one would argue with”² have made their way into the data bank.

Of 562 data bank files containing nucleic acid coordinates, we found that 34 contained reversed configurations (Table 1) of at least one chiral centre (Fig. 1). Incorrect configurations at C1', C3' and C4' are detectable in both NMR and crystal structures. C2' represents a special case for DNA structures where H2' and H2'' are included. Errors in the naming of H2'/H2'' atoms would be irrelevant in X-ray structures but are significant in NMR structures of DNA because H2' and H2'' are almost always stereospecifically assigned. In the case of RNA, the correct chirality of C2' would be equally significant in both X-ray and NMR structures.

In 25 further coordinate files, not listed

Table 1 Chirality problems in nucleic acid coordinate sets from the Brookhaven Protein Data Bank

PDB entry code	Total number of nucleosides	Chirality errors		Method	PDB entry code	Total number of nucleosides	Chirality errors		Method
		1', 3', 4'	2'				1', 3', 4'	2'	
106D	96	0	8	NMR	1DSD	16	0	1	NMR
108D	640	0	20*	NMR	1HRY	16	2	1	NMR
143D	132	21	20	NMR	1HRZ	560	70	35	NMR
149D	294	0	147	NMR	1LBG	84	7	0	X-ray
170D	22	0	10	NMR	1QDF	15	7	15	NMR
171D	24	0	12	NMR	1QDG	15	7	0	Theory
173D	16	1	0	X-ray	1QDH	15	7	0	NMR
186D	168	9	5	NMR	1RCS	600	36	0	NMR
1ARA	136	34	87†	NMR	1URN	55	0	2†	X-ray
1BUF	12	2	0	NMR	203D	640	0	60*	NMR
1D31	26	1	0	X-ray	204D	640	20	40*	NMR
1D42	80	1	0	NMR	228D	88	9	73	NMR
1D70	64	1	0	NMR	229D	14	2	7	NMR
1D98	24	1	0	X-ray	28DN	8	1	0	X-ray
1D99	24	1	0	X-ray	2DA8	96	0	2	NMR
1DDP	200	0	20*	NMR	5BNA	24	1	0	X-ray
1DSC	16	0	1	NMR	5ZNA	32	0	16	Theory

A chiral centre was designated wrong if the dihedral angle defined by its four ligands – or three ligands and the central atom in cases where no hydrogen coordinates are given – was found to have the opposite sign of the correct configuration. A complete, detailed table of chirality problems is available at <http://www.mbi.ucla.edu/people/peter/chiral.html>.

*In these cases the majority of H2'/H2'' configurations are inconsistent with the convention as given in ref. 3. The number given represents configurations opposite to the nomenclature used within these entries. Coordinate files where all H2'/H2'' configurations are reversed throughout are not listed.

†RNA, number of flipped O2' atoms.

in Table 1, the naming of H2'/H2'' was reversed throughout compared with the IUPAC-IUB convention^{3,4}. Whether these cases are merely in discrepancy with the convention in a particular file or inconsistent with the underlying experimental data would depend on whether the same nomenclature was used for the determination of the stereospecific resonance assignment in each case.

We also observed inconsistencies in the naming of the amino protons, which are distinguishable even when they are not involved in hydrogen bonds simply by their *cis/trans* positions with respect to other base atoms. We disregarded inconsistencies in the naming of the diastereotopic atom pairs H5'/H5'' and O1P/O2P as they are generally not relevant to the experimental results in either NMR or X-ray structures. However, these atoms should be consistently named for accurate structure comparisons by root mean square deviations of atomic positions.

The overall topology of most affected structures would probably not change substantially on correction of their chirality problems and re-refinement. Nevertheless, these errors are significant both because the structures are stereochemically wrong and because of the importance of ion and hydrogen bonding interactions with the phosphodiester backbone which stabilize the structures of nucleic acids.

We note that current refinement programs and their parametrization for nucleic acids do not always prevent this type of avoidable error, especially when high-temperature simulated annealing protocols are used. The higher number of errors found in

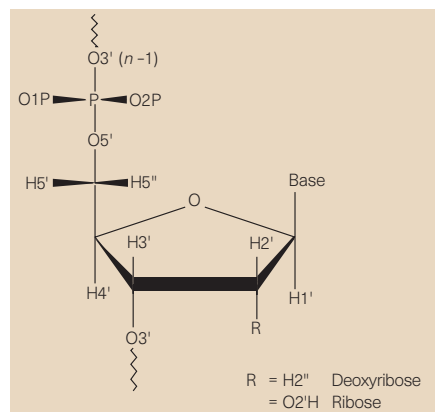


Figure 1 Stereochemical nomenclature of a nucleotide. Carbon atoms are numbered from that bound to the base.

the NMR structures compared with the X-ray structures does not reflect a weakness of NMR experiments, but rather depends on problems in the refinement protocols.

It might be reasonable to test all structures for rigorous adherence to the existing conventions of stereochemical nomenclature^{3,4} at the time of deposition into the data bank.

Peter Schultze, Juli Feigon

Department of Chemistry and Biochemistry,
University of California,
Los Angeles, California 90095-1569, USA
e-mail: peter@mbi.ucla.edu; feigon@mbi.ucla.edu

1. Hooft, R. W. W., Vriend, G., Sander, C. & Abola, E. E. *Nature* **381**, 272 (1996).
2. Jones, T. A., Kleywegt, G. J. & Brünger, A. T. *Nature* **383**, 18–19 (1996).
3. IUPAC-IUB Joint Commission on Biochemical Nomenclature *Eur. J. Biochem.* **131**, 9–15 (1983).
4. Liebecq, C. *Biochemical Nomenclature and Related Documents* (Portland, London, 1992).

Medicine in the marketplace

21st-Century Miracle Medicine: RoboSurgery, Wonder Cures, and the Quest for Immortality

by Alexandra Wyke

Plenum: 1997. Pp. 352. \$26.95, £16.33

Edward S. Golub

The thesis of this book, which is directed to educated nonscientists, is that twentieth-century medicine has failed because, despite all the money spent on health care, people are “manifestly no less sick than they were three decades ago”. According to Alexandra Wyke, a respected journalist who writes for *The Economist* magazine, the public knows this and demands technological solutions.

Although her book's subtitle promises the quest for immortality, Wyke's twenty-first-century miracle medicine, by effecting the virtual disappearance of the major diseases of the late twentieth century, will add a decade to life. The vision of the future in which these goals are achieved will be controversial, so it is appropriate that we should ask whether twentieth-century medicine really is a failure. Can the technology Wyke describes really extend life expectancy a decade and eliminate disease as we know it? And, if it can be done, should it be done?

Wyke's claim that twentieth-century medicine has failed is based on the fact that heart disease, cancer, diabetes and other chronic diseases are increasing — using 1970 as the benchmark. But when we look at the beginning of the century things look quite different: from 1900 to the present infant mortality dropped tenfold, and life expectancy doubled.

Wyke is actually responding to the profound change in demographics of the twentieth century. As I pointed out in *The Limits of Medicine* (Times Books/ University of Chicago Press; 1994 and 1997) we controlled and cured infectious diseases through sanitation and antibiotics. What resulted were chronic diseases of an ageing population that we are learning to control through drugs and lifestyle change. So Wyke and I disagree that twentieth-century medicine is a failure; but what about the elimination of chronic diseases through technology?

Wyke, who has a PhD in biochemistry, travelled the world interviewing scientists and industrialists to discover what technology is about to enter the pipeline, and concludes that the “miracles” of twenty-first-century medicine will come about through scientific advances in informatics and genomics.

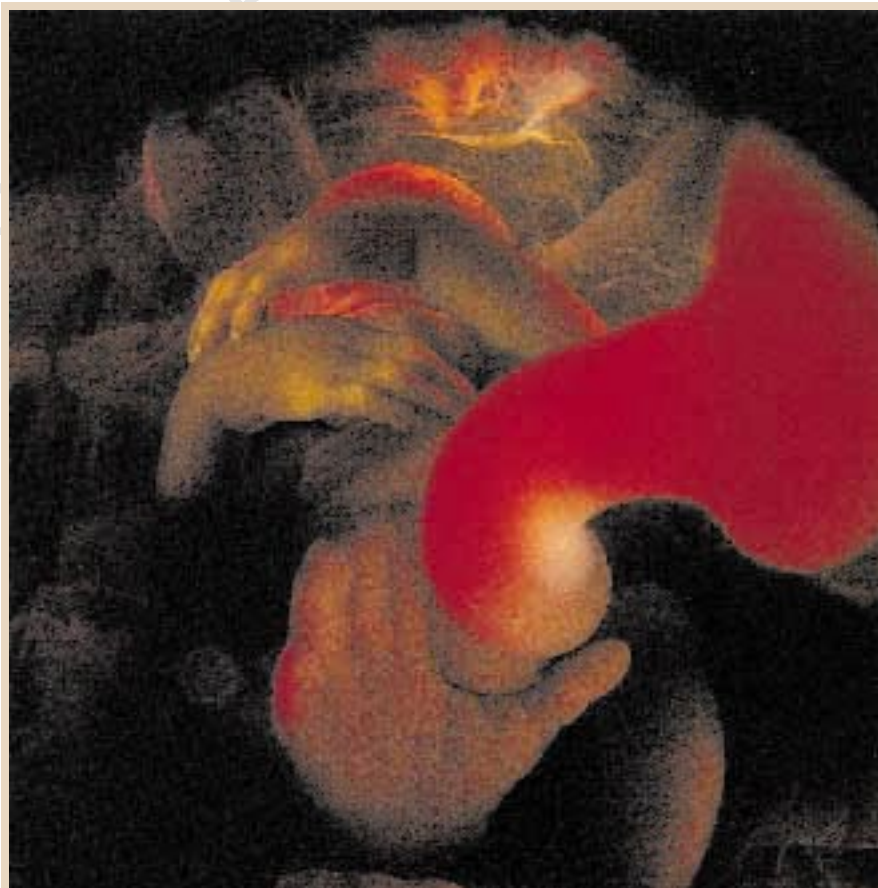
The computerized medical records, online access to databases for diagnosis, and computer-assisted microsurgery using the

tools of virtual reality that she predicts seem quite reasonable. One winces at the thought of nerve-sparing prostate surgery done by a robot, but there seems little doubt that informatics and robotics will continue to change the way we handle information and how we work. How they will change medicine at a fundamental level depends on what we ask them to do. Wyke thinks we will ask them to change our genes.

She writes: “By examining and manipulating genes... biological scientists believe the way is open for them to tackle and seemingly eliminate most of the diseases that afflict the world — including ones that have yet to appear”, because the structure of DNA has “provided the means to study the bible by which humans live, opening up the potential to register the conditions under which this good book can become desecrated — when, in other words, we fall sick and die”. That offers “the possibility of little

less than the eradication of disease itself”.

She cites Eric Lander, who predicts that by 2005 the sequences of all human genes will be known, but extrapolates from this that the mechanisms by which they cause disease will be worked out by 2050. It is significant that the part of the book that deals with predictions for computers and robotics contains direct quotations from the scientists and industrialists who are pushing their products, but I could find no direct quotations from the biologists involved in the genetics. Wyke does not even mention the views of the growing number of geneticists who argue that phenotypes can be the result not only of linear interactions between genes and gene products but also of emergent properties that must ultimately be studied using the new science of complexity, nonlinear dynamics and chaos theory. This is very important, because her book is likely to be widely read and to leave



Life before birth

The Swedish photographer Lennart Nilsson continues to stun with his prenatal images of humans and animals. This one, of a chimpanzee fetus clutching its umbilical cord four months before birth, wins an entry in *World Press Photo Yearbook 1997* edited by Ben

Ten Berge and Kari Lundelin. Selected from over 3,500 entries from around the world, the 200 photographs in the book were judged by the World Press Foundation as the best in visual reportage last year. Thames and Hudson, £9.95 (pbk).

the impression that this crudest form of genetic determinism is representative of the thinking of responsible biologists.

Wyke and I clearly disagree on the likelihood of science leading to the elimination of chronic disease by 2050. But we must still face the third, and for me the most important, question — even if the science were sound and her vision could be realized, should it be? Given the public response to genetically engineered food and the cloning of Dolly, scientists must be very sensitive to the extra-scientific aspect of science and technology. The chapter entitled “The Year 2050” depicts a world in which the benefits of the revolution are in place, and it is a world from our worst science-fiction nightmares.

People are connected to CompuDoc computers that scan their bodies and connect them to MediMechanics who appear as smiling, white-coated, holographic images and order drugs to be delivered in dosages determined from analysis of the patient’s genes held in the health corporation’s computer. Or distance robotic surgical procedures — RoboSurgery — are carried out on people in the comfort of their Orwellian homes.

In a stunning chart that compares the pattern and nature of death in 1990 with what is predicted for 2050, we see that in this brave new medical world only 25 per cent of deaths will be from heart disease, cancer, diabetes and other diseases that killed more than 85 per cent of the population in 1990. This should be good news — the dream of high-tech “miracle” medicine fulfilled. But the chart for the year 2050 shows that 25 per cent of the deaths of a population presumably living well into their nineties will be by euthanasia and suicide, and another 20 per cent from homicide.

This, I fear, is what the lay public will focus on and ask why nonagenarians will want to end their lives in the presence of such “miracles”. They will assume that science is really offering the public a world in which the technology of medicine makes life unbearable for a quarter of the population. Ordinary people and legislators who fund science will find scant comfort from Wyke’s statement that genetics can control the high murder rate because “2050s society may hold in its grasp the ability to alter genes controlling overpowering tendencies to murder and rob”.

But Wyke sees the social impact of this future quite differently. In the very next chapter she rails against the haughtiness of the medical profession for arrogating medicine to itself, and against the intrusiveness of regulatory agencies such as the US Food and Drug Administration for interfering with the ability of the market to reject bad drugs. “Medicine, indeed, appears destined to roll along the very same path as any other con-

sumer-led industry, with its hitherto dominant components, in this case doctors and governments, being relegated to mere participants catering to the public’s various needs.”

Wyke thinks that the invisible hand of the market will manage these “miracles”. But I doubt whether Adam Smith himself would have thought that consumers could have any power to influence the corporations that would run the computers containing all the genetic, psychological and economic data. The hand of the medical market Wyke foresees is very visible indeed, and may be part of the reason for a quarter of the population choosing suicide and euthanasia.

The most perplexing thing about her book is that Wyke knows this. The last two chapters are devoted to the role of caring, human contact between patient and doctor, the unexplained power of the placebo, and the fact that people are turning away from the coldness of scientific medicine as it is too often practised in favour of alternative medicine. She is clearly aware of the danger of the loss of privacy in an electronic world. I reread these chapters several times trying to reconcile their content with the vision of the computerized, roboticized twenty-first-century medicine that is the central message of the book.

I began to suspect that the vision of CompuDocs, RoboDocs, vast computer databanks containing the most intimate information about our lives, genes replaced to make us ‘normal’, and finally the grim vision of people queueing up to end their lives, was too much for her.

But I am probably wrong, and my suspicion may be only a reflection of the fear I have that *21st-Century Miracle Medicine* will frighten its audience more than it will excite them, and will blind them to the great potential good science can bring to medicine. The mysticism of Deepok Chopra and the alternative medicine of Andrew Weil are already increasing in popularity, and growing numbers of people are turning away from scientific medicine to New Age healing. Scientific medicine is certainly capable of using the enormous insights we are gaining into normal function and disease to improve people’s lives without enslaving them to an all-intrusive, computer-driven, medical delivery system.

The control people want over their lives, and medicine that uses technology to enrich rather than enslave that Wyke talks about in the last chapters, will not come from the uses of technology she describes in earlier chapters. The book describing that vision of twenty-first-century medicine remains to be written. □

Edward S. Golub is at the Pacific Center for Ethics and Applied Biology, Solana Beach, California 92075, USA.
e-mail: egolub@esga.com

War of the rosé

Science, Vine, and Wine in Modern France

by Harry W. Paul

Cambridge University Press: 1997. Pp. 355.
£45, \$64.95

Bernadette Bensaude-Vincent

Having been born into a family of vine-growers, near Montpellier in the south of France, I remember well that the history of our village was divided into two periods — before and after the phylloxera that devastated the vineyards from 1875 to 1900. This plague deprived the region of its main source of revenue and its effects entered the collective memory of our village in the same way as the First World War later left its mark. From Harry Paul’s fascinating account of the episode, we learn that the plague was, in fact, a war — a multifaceted war.

First, it was a war of men against an invisible enemy, a small insect known as *Phylloxera vastatrix*. To help the farmers, who initially tried to drown the pest by flooding the vineyards, troops of scientists were mobilized. A national academic committee, presided over by the chemist J. B. Dumas, recommended the use of potassium sulphocarbonate as an insecticide. The chemical warfare proved ineffective and the vines, already weakened by diseases such as mildew, died.

Plant scientists from the school of agriculture in Montpellier advocated their own programme of replacing the dead vines with French ones grafted on to American rootstocks. There followed a scientific controversy between this government-approved programme and an alternative solution of replacing the dead vines with direct-production hybrids that were more resistant to insects and would therefore not require extensive use of chemicals to protect them.

This second war — between the pro-grafting ‘Americanists’ and the partisans of hybridization — was conducted through laboratory experiments which, in turn, generated a secondary conflict between laboratory scientists and large-scale experimenters who argued that conclusions inferred from ‘pot-vines’ were not valid for vines in the field.

The pro-grafting school of Montpellier, supported by the government, spread its message among farmers through journals and popular conferences. The hybrid camp replied by establishing its own journals and by mounting a vigorous anti-academic campaign against ‘official science’. Although hybrids still flourished in a seventh of France’s vineyards until 1940, by the 1910s the alternative grafting programme was already considered the winning solution and the one founded on scientific certainties.



Battle fields: Harry Paul unearths a social tension between small producers and owners of big vineyards in early twentieth-century France.

Paul's penetrating analysis of this controversy shows the underlying social tension between small producers who favoured the hybrid programme and owners of big vineyards who favoured the riskier solution of grafting which gave higher-quality wines.

After analysing the misadventures of vines, Paul turns to the science of wine. He describes the emergence of oenology as a cross-disciplinary science, one that, despite the leading role of chemists in its development, has never been considered a part of applied chemistry. Most French oenologists worship a trinity of founders of their discipline: Jean-Antoine Chaptal, the pioneer in the 1810s; Louis Pasteur, the 'father of oenology' from the mid-nineteenth century; and Jean Ribèreau-Gayon, the promoter of modern oenology in the 1930s.

In presenting this standard account, Paul keeps an ironic distance yet he offers no real revision of it. To construct an alternative historiography of oenology would require further investigations of neighbouring disciplines, including botany and chemistry. In particular, the alliance between producers and academic scientists needs to be understood within the context of the development of agricultural chemistry and agricultural stations throughout France.

By contrast, Paul is more innovative in his geographical approach to the development of oenology where he emphasizes regional styles of science. The wines from Champagne, Burgundy and Bordeaux, and the ordinary *vins de table* from Languedoc, each called for different sciences. Although the school of Montpellier eventually became the leader of viticulture and that of Bordeaux the leader of modern oenology, the various schools rivalled one another and sometimes entered into controversies. One of the most memorable disputes con-

cerned the effects of the method of heating wine — known as pasteurization because it was recommended by Pasteur — on the quality of the wine.

A fascinating issue emerges from Paul's approach: how can one articulate a scientific knowledge of wines when their main characteristics and values are dependent on local circumstances? How, in other words, can one apply universal laws and rules to a product of the local terrain and climate? Regrettably, however, this issue is never explicitly discussed by Paul, even though it seems to dominate his book's structure.

Paul is more concerned with another challenge of the science of wine: how can one quantify qualities of taste? He carefully describes the complex process of wine making and the transformations it has undergone. He emphasizes the dream of scientists of the late nineteenth century that wine making could be scientifically controlled. Repeated attempts to identify the cause of the individual taste of each *cru* in the nature of the yeast were defeated by "the caprices of the yeast".

It was thanks to the development of physical chemistry, by the turn of this century, that scientific control of the wine-making process was made possible. The ionic approach developed in the 1930s by Ribèreau-Gayon, in particular his careful study of oxidation at various stages of wine processing and in the ageing of wine, reinforced the programme of the "apostles of chemistry".

Paul is reticent about today's attempts to produce high-technology wines. He concludes that since the 1930s oenology has done more to improve the standardization and preservation of wines for commercial purposes than to improve their bouquet. But this is not a kind of romantic nostalgia for the

original product, because Paul emphasizes strongly that wine has never been a natural product, but rather has always been an artefact. That is the personal opinion of a connoisseur of French wines.

This book will doubtless interest all wine connoisseurs and entertain scientists. It is, moreover, extremely useful in turning the attention of historians' towards a neglected science, one whose hybrid status between science and technology, and between cognition and commerce, is fascinating. □

Bernadette Bensaude-Vincent is in the Département de Philosophie, Université de Paris-X, 92001 Nanterre, France.

A Darwinian Universe?

The Life of the Cosmos

by Lee Smolin

Oxford University Press/Weidenfeld and Nicolson: 1997. Pp. 320. \$30, £20

George Ellis

Lee Smolin presents an intriguing attempt at a cosmological synthesis of three of the major discoveries of modern science — the evolution of the Universe from a hot Big Bang, the quantum nature of fundamental physics, and the Darwinian theory of evolution by natural selection. It is the in-depth development of the latter aspect that makes the book unique.

The author takes various themes from the recent literature on cosmology — the anthropic issue (why is the Universe of such a nature that it admits the existence of life?), chaotic inflation (proposing many separate expanding regions in a self-reproducing chaotic Universe, with a process of 'mutation' leading to variation of fundamental physical constants) and quantum cosmology (investigating the initial conditions of the Universe) — and weaves from them a proposal for a unified view of the Universe. This is based on two overriding principles: first, that all fundamental physics in the end is relational, and second that evolutionary selection (through mutation leading to differential reproduction rates) is the only physically based process that is in principle capable of generating new kinds of self-organized non-equilibrium structures.

He therefore arrives at the concept of the Universe itself evolving by a process akin to Darwinian evolution. Many successive collapses of stars into black holes generate new expanding regions of the Universe with slightly different physics. The regions that come to dominate are those where local physics is such that production of black holes (and hence of new baby universes) is most likely. These universes are then claimed to be likely to allow the evolution of life, and hence our own existence.

So this is a route whereby not merely complex structures such as life come to arise in a natural way, but the laws of physics themselves also become adapted to make possible this existence of complex structures.

The case is argued with skill and sophistication. The book is carefully written for the nonspecialist and is a pleasure to read, with many excellent descriptions of modern scientific ideas that will convey the excitement of science to the nonscientist. Because the book aims to deal with the 'big questions' of fundamental importance, it considers issues in science, metaphysics and religion.

The book deals with a vast spectrum of science. What is proposed is highly speculative, but then that holds true for much of present-day theoretical physics. The central mechanism envisaged is conceivable but incomplete: we do not yet have a well-developed proposal as to how collapse to a black hole can be reversed to expansion into a new region. The final sections on relational approaches to fundamental physics are somewhat vague. But this is all fine because, in contrast to many recent popularizations of science, this book makes clear what is well-established theory and what is speculative, with one important exception: it is not demonstrated that the physics that would lead to maximal star formation would also necessarily allow the complex molecular chemistry of life. This is assumed rather than argued; it certainly needs further justification. But overall the science is interesting, reasonably plausible if not proved, and well argued.

The underlying metaphysics is much less satisfactory. Again, it is very helpful that, unlike many writers, Smolin makes

explicit his metaphysical aims. But his hope of attaining coherent laws of physics from pure chaos or from nothing is illusory, as has been pointed out by Paul Davies. Smolin seems unaware of the body of literature in cosmology. He does not refer, for instance, to Dirac, Bondi, Sciama, Wheeler and Feynman, Brans and Dicke, or Hoyle and Narlikar.

This literature makes clear that all one can sensibly achieve in following this route is to replace some constancy in present-day physics (for example, the value of the gravitational constant) by a variability that is itself controlled by another law which is constant and unchanging; for, without this, there is nothing one can say. In the end, all one can do is replace specific physical laws by higher-level laws; the fundamental metaphysical situation is unaltered. The author's proposals are no exception. Even if he does not acknowledge it, they are underpinned by assuming continued validity of physical ideas that can remain true only if they reflect continuing unchanged physical behaviour that can be described by unchanging (higher-level) physical laws — which is what he is trying to avoid.

His final metaphysical proposal — to derive physics that cannot be interpreted as a whole by an observer "outside of the world" — is in my view unnecessary and uninteresting (although it does show that most attempts at a fundamental physical theory do not satisfy his proposed principle). His motivation comes from a desire to exclude a concept of God from his metaphysical picture. This is where his book is weakest.

It is not necessary to refer to religious

issues in considering the fascinating physical ideas that are the main burden of this book. However, if one chooses to do so (and the author does — there is a chapter entitled "Philosophy, Religion, and Cosmology") then one has an obligation to do so properly. In a number of recent writings by scientists, there is a catastrophic decline in academic rigour and quality of argument when referring to these issues. Smolin is more sophisticated than many, but nevertheless does not follow the basic principles one would expect of anyone wishing to make a serious contribution to thought in the science-religion debate.

Moving to a new area of science, one would expect a researcher to be aware of the current debate and in touch with recent relevant literature (for example, *Religion and Science: History, Method, Dialogue* edited by M. Richardson and W. Wildman, Routledge, 1996, and references therein); to follow the basic principles of critical thought, such as looking seriously at the evidence supporting opposing views; and to respond to the best available thought in the opposing camp, and not to some ersatz argument that no sensible person would support. When it comes to the science-religion issue, Smolin does none of these things. He does not, for example, refer to Torrance, Ian Barbour, Soskice, Peacocke, Polkinghorne, Murphy, Bowker or Ward.

So the comments made in this area are disappointing, and not at the same level as the rest of the book. This reflects a broader theme: if scientists wish to enter effectively the debate about the ultimate meaning of cosmology and its relation to human life, they need to take humanity seriously as well as taking scientific data seriously. The evidence they consider needs to refer to the deepest aspects of human life — pain and joy, love and war, morality and ethics — and not merely to the fact that we are made of carbon-based molecules organized in complex ways.

The approach presented in this book in the end does not take such data seriously — when theorizing about ultimate meaning, only the physical data and theories are taken as significant. This is surprising in view of many vignettes in the book showing a much wider appreciation of life than can be encompassed in the theoretical physicist's highly simplified physical models of reality which largely ignore emergent order and meaning.

Despite this criticism, this thought-provoking book is much more broad-ranging and considered than most of those that tackle fundamental issues of cosmology. It is well worth reading, and is highly recommended to scientists and nonscientists. □

George Ellis is in the Department of Mathematics and Applied Mathematics, University of Cape Town, Rondebosch 7701, South Africa.

New in paperback

Lise Meitner: A Life in Physics

by Ruth Lewin Sime

University of California Press, \$16.95

"A detailed new biography.... Written by the American chemist Ruth Lewin Sime, it is clearly a labour of dedication, if not of love; it seems to have taken her 20 years to write. It is also a partisan book. Sime clearly does not like Meitner's German colleagues such as Heisenberg, von Weizsäcker and Meitner's life-long friend and co-worker Otto Hahn. As I don't much like them either, her point of view does not bother me at all, although it may trouble some readers", wrote Jeremy Bernstein in *Nature* 380, 33 (1996).

Bombardier Beetles and Fever Trees: A Close-up Look at Chemical Warfare and Signals in Animals and Plants

by William Agosta

Helix Books (Addison-Wesley), \$13

"A lucid account of the chemistry behind the natural world. As part of the current renaissance in popular science books written by leading scientists, it may help to do for chemistry what others have done for evolution and genetics", wrote Tristram D. Wyatt in *Nature* 381, 289 (1996).

Feynman's Lost Lecture: The Motion of the Planets Around the Sun

by David L. Goodstein and Judith R. Goodstein

Vintage, £6.99

"The book goes over some well-trodden history, from Copernicus through Newton to Einstein, and contains reminiscences about Feynman.... The core of the book is a step-by-step account of [Feynman's] argument that will be accessible to any science student, from sixth-form (high-school) level upwards", wrote Paul Murdin in *Nature* 380, 680 (1996).

Signalling through the lipid products of phosphoinositide-3-OH kinase

Alex Toker & Lewis C. Cantley

When a stimulatory agonist molecule binds at the exterior of the cell membrane, a second messenger transduces the signal to the interior of the cell. Second messengers can be derived from phospholipids in the membrane by the action of the enzymes phospholipase C or phosphoinositide-3-OH kinase (PI(3)K). PI(3)K is a key player in many cellular responses, including the movement of organelle membranes, shape alteration through rearrangement of cytoskeletal actin, transformation and chemotaxis. But how PI(3)K mediates these responses is only now becoming clear.

The discovery of phosphoinositide-3-OH kinase some nine years ago suggested that there was a new intracellular signalling system involving lipid second messengers. An activity capable of phosphorylating the D3 position of phosphatidylinositol to produce phosphatidylinositol-3-phosphate (PtdIns(3)P) was first detected in a complex with viral Src and the middle-T antigen of the polyoma virus¹. At about the same time, PtdIns(3,4,5)P₃, a lipid not previously known to exist, was detected in activated neutrophils². The enzyme responsible for producing these lipids was later purified and shown to phosphorylate PtdIns(4)P and PtdIns(4,5)P₂ to produce PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. Several other observations indicate that these lipids act as second messengers: they are nominally absent in unstimulated cells, and rapidly accumulate after stimulation with various agonists; phosphatidylinositol-3-OH kinase (PI(3)K) lipid products are not substrates for phospholipases, and inhibition of the production of these lipids results in inhibition of many acute cellular responses. Although PI(3)K has long been implicated in a variety of cellular responses to stimuli, three recent discoveries have intensified the interest in signalling through this pathway. First, activation of PI(3)K has been shown to prevent apoptosis in several cell types³; second, a retrovirus-encoded PI(3)K was found to cause haemangiosarcomas in chickens and to transform fibroblasts⁴; and third, mutations in a *Caenorhabditis elegans* PI(3)K gene cause a threefold increase in the lifespan of the adult⁵.

Since the initial discovery of PI(3)K and its lipid products, cDNA cloning efforts have led to the discovery of additional PI(3)K family members, some with restricted substrate specificity (Fig. 1). PI(3) kinases such as the first described p85/p110 heterodimer are distinct from other enzymes such as Cpk, a C2-domain-containing PI(3)K which can only use PtdIns and PtdIns(4)P as substrates and so offer

a distinct pathway for the production of PtdIns(3,4)P₂. Importantly, the p110 catalytic subunit of certain PI(3) kinases has a binding site for the small GTP-binding protein Ras, and PI(3)K is therefore a direct effector of Ras⁶. There are also distinct enzymes such as the yeast Vps34, which can only produce PtdIns(3)P, and are thus named PtdIns(3) kinases. The regulation of the metabolism of D3 phosphoinositides is further complicated by the discovery of phosphatases that can specifically remove the D3, D4 and D5 phosphates (Fig. 1). Enzymes such as SHIP (Src-homology-2 (SH2)-containing inositol 5'-phosphatase) can remove the 5'-phosphate from PtdIns(3,4,5)P₃ and may control the ratio of PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂. It is clear that the metabolism of the lipid products of PI(3) kinases is complex and highly regulated within cells. PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ are acutely regulated in agonist-stimulated cells, whereas PtdIns(3)P is constitutively present. The implication is that there are proteins within the cell that specifically recognize and interact with distinct D3 phosphoinositides in a regulated manner.

Targets of D3 phosphoinositides

Three important advances in the last few years have accelerated the discovery of proteins that interact differently with phosphoinositides. The first was the discovery of the fungal metabolite wortmannin as a potent inhibitor of the PI(3)K family of enzymes. Wortmannin has proved a valuable reagent for studying PI(3)K-dependent responses, but it is now clear that wortmannin has other targets in the cell, with activities that are inhibited by relatively low concentrations of the drug. The second advance has been in genetic studies which implicate phosphoinositide kinases and their products in signal transduction. Several laboratories have generated mutants of the platelet-derived growth factor (PDGF)- β receptor in

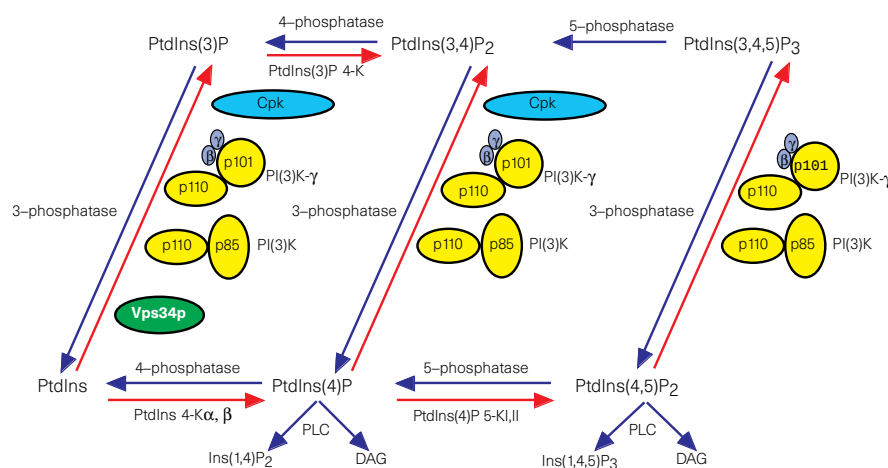


Figure 1 Pathways for phosphoinositide synthesis. The enzymes that synthesize the various phosphoinositides are indicated. Three classes of PI(3)K enzymes exist: the class I enzymes (yellow) can use PtdIns, PtdIns(4)P or PtdIns(4,5)P₂ as substrates; class II enzymes (blue) phosphorylate PtdIns and PtdIns(4)P; and class III enzymes (green) can only phosphorylate PtdIns. PtdIns(4,5)P₂ is of particular importance: hydrolysis by phosphoinositide-specific phospholipase C (PLC) generates the two second messengers, diacylglycerol (DAG) and Ins(1,4,5)P₃, and phosphorylation by PI(3)-kinase generates the putative second messenger PtdIns(3,4,5)P₃. PtdIns(3,4)P₂ can be generated through the phosphorylation of PtdIns(4)P by a PI(3)K (Cpk, C2-domain-containing PI(3)K), as well as by dephosphorylation of PtdIns(3,4,5)P₃. In mammalian cells, D3 phosphoinositides are degraded by phosphatases that convert them back to PtdIns, PtdIns(4)P or PtdIns(4,5)P₂.

which critical tyrosine residues that mediate direct association with signalling molecules have been mutated to phenylalanine. On the basis of these studies, phospholipase C (PLC)- γ and PI(3)K have been implicated in PDGF-dependent mitogenesis. Constitutively active and dominant-negative PI(3) kinases have gone a step further in describing targets and pathways activated downstream of this enzyme. Receptor mutants and activated PI(3) kinases have therefore provided evidence for a number of proteins downstream of PI(3)K. The remaining challenge has been to describe which of these are direct targets of phosphoinositides. This critical step has been achieved by the third advance, the availability of a variety of synthetic analogues of phosphoinositides for biochemical studies. By using these reagents, a number of proteins/enzymes have been found to bind phosphoinositides *in vitro*, and in some cases stereospecific interactions have been demonstrated.

Src-homology-2 domains. The SH2 domains of Src and the p85 regulatory subunit of PI(3)K have been shown to bind specifically to PtdIns(3,4,5)P₃ (ref. 7). This association could be competed with both phenyl phosphate and phosphotyrosine-containing peptides, indicating that phospholipid binding and phosphopeptide binding are mutually exclusive. Using SH2 mutants of Src, the arginine residue at position 178 (in the conserved FLVR motif) was shown to be critical for the phosphotyrosine interaction, but not for PtdIns(3,4,5)P₃ binding, suggesting that different residues are responsible for coordinating the phosphotyrosine and phospholipid moieties⁷. The *in vivo* relevance of this result is supported by the observation that inhibition of PtdIns(3,4,5)P₃ production results in enhanced association of the p85 subunit of PI(3)K with tyrosine-phosphorylated insulin receptor and IRS-1. These results suggest that PtdIns(3,4,5)P₃ binds directly to the SH2 domains of p85 and causes dissociation of PI(3)K from tyrosine-phosphorylated proteins. It is also likely that PtdIns(3,4,5)P₃ could specifically recruit other SH2-containing proteins to the membrane (Fig. 2).

Pleckstrin-homology domains. Of particular relevance to phosphoinositide signalling is the pleckstrin-homology (PH) domain, which can mediate either protein–protein or lipid–protein interactions, or both. Several PH-domain-containing proteins, including pleckstrin, phospholipase C- δ 1, dynamin, SOS and the β -adrenergic receptor kinase have been shown to bind to PtdIns(4,5)P₂ and/or Ins(1,4,5)P₃ directly. The crystal or solution structures for a few PH domains bound to Ins(1,4,5)P₃ have been determined. More recent studies have addressed the binding of PH domains to D3 phosphoinositides, and indicate that different PH domains have specificity for distinct phosphoinositides.

Akt/PKB. The Akt/PKB protein Ser/Thr kinase (also known as RAC- α) lies downstream of PI(3)K in agonist-stimulated cells^{8,9}. This protein has an amino-terminal PH domain, and deletion of this domain blocks the ability of some agonists (such as PDGF)⁸ but not others (such as insulin)¹⁰ to activate Akt *in vivo*. A single point mutation (R25C) in the PH domain of Akt has been shown to impair activation in response to PDGF and in cells expressing activated PI(3)K¹¹. Recent studies have also indicated that PtdIns(3,4)P₂ can directly stimulate Akt activity^{11–13}. PtdIns(4,5)P₂ does not activate Akt and PtdIns(3,4,5)P₃ has no effect, or even inhibits activity under some conditions. This activation correlates with the ability of the purified protein and the isolated PH domain of Akt^{11,13} to bind to PtdIns(3,4)P₂ with higher affinity than it binds to PtdIns(4,5)P₂ or PtdIns(3,4,5)P₃. PtdIns(3,4)P₂ binding also induces dimerization of Akt molecules¹¹. Although PtdIns(3,4)P₂ binds to and activates Akt, it is clear that additional regulatory mechanisms are required for full activation *in vivo*. A new kinase called PtdIns(3,4,5)P₃-dependent protein kinase-1 (PDK1), which phosphorylates and activates Akt *in vitro*, has recently been purified¹⁴. PDK1 is itself directly activated by both PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. An additional, as-yet undescribed protein kinase, which may also be regulated by PI(3)K lipids, is predicted to phosphorylate additional sites in the catalytic domain of Akt. The

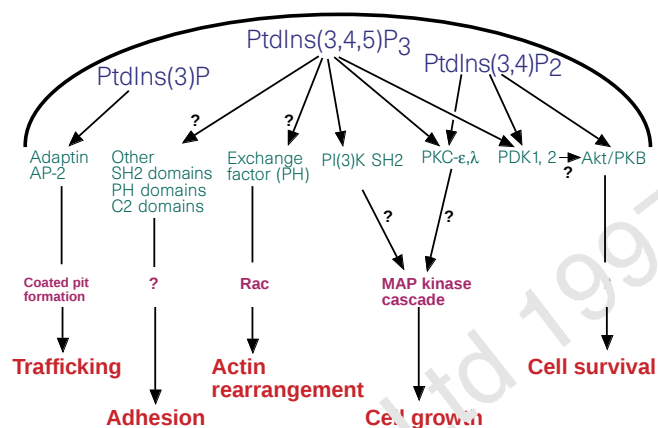


Figure 2 Proteins known to bind phosphoinositides *in vitro*: possible roles in cellular responses. Accumulation of PtdIns(3)P, PtdIns(3,4)P₂ and/or PtdIns(3,4,5)P₃ at the membrane is likely to recruit multiple signalling molecules that initiate secondary signalling cascades. In some cases, the direct targets of D3 phosphoinositides may feed into other signalling cascades (such as the MAP kinase cascade). The diversity of proteins that bind to these lipids may provide an explanation for how PI(3)K family members regulate so many diverse physiological functions.

model that is emerging is that localized production of PtdIns(3,4)P₂ at the plasma membrane recruits Akt, facilitating dimerization and leading to a partial activation. This may lead to a conformational change in Akt, exposing the enzyme to upstream regulatory protein kinases which are themselves activated by PI(3)K lipid products. Once phosphorylated, Akt is locked into the fully activate conformation. In this model, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ play a critical role in the activation of Akt (Fig. 2). However, there are other pathways for Akt activation that bypass the PI(3)K requirement¹⁵. The regulation of Akt/PKB *in vivo* is therefore highly regulated and complex, and may depend on multiple redundant signals.

Several biological responses have now been attributed to Akt, including cell survival and gluconeogenesis. A number of targets of Akt have been described, including glycogen synthase kinase 3 and p70 S6 kinase. In summary, Akt is an important downstream effector that mediates some of the biological responses of PI(3)K. Btk. The non-receptor Bruton's tyrosine kinase (Btk) is involved in B-cell development, and several mutations in its PH domain are responsible for X-linked immunodeficiency in mice (Xid) and X-linked agammaglobulinaemia (XLA) in humans. Recent studies have demonstrated that the PH domain of Btk has high affinity for PtdIns(3,4,5)P₃ and Ins(1,3,4,5)P₄, but binds with relatively low affinity to PtdIns(3,4)P₂ and PtdIns(4,5)P₂ (refs 16, 17, 22). The Xid mutation R28C in the Btk PH domain reduces the affinity for PtdIns(3,4,5)P₃. This mutation is analogous to the R25C mutation in Akt that abolishes PtdIns(3,4)P₂ binding and activation. These data suggest that there is a link between D3 phosphoinositides and B-cell differentiation. Although a link between PI(3)K and Btk has not yet been demonstrated *in vivo*, PtdIns(3,4,5)P₃ could function either to activate the enzyme directly or to localize it at the membrane in proximity to an upstream regulating kinase (Fig. 2). Ins(1,3,4,5)P₄ could act to release Btk from the membrane.

Grp1. Grp1 (general receptor for phosphoinositides)¹⁸ is a PH-domain-containing protein that is highly related to cytohesin-1 (ref. 19) and ARNO (ref. 20), all three of which have amino-terminal PH domains. The PH domain of Grp1 has high affinity for PtdIns(3,4,5)P₃ but relatively low affinity for PtdIns(3,4)P₂ and PtdIns(3)P. The Sec7 domain of the related protein ARNO stimulates exchange of GDP and GTP on ARF-1, and the PH domain mediates PtdIns(4,5)P₂-dependent stimulation of this activity²⁰. Curiously, the N-terminal domain of the highly related protein

cytohesin-1 (including the Sec7 domain) was implicated in binding to and activation of $\beta 2$ integrins¹⁹. Because cytohesin-1, ARNO and Grp1 are highly related, it is likely that all of these proteins bind PtdIns(3,4,5)P₃ and act as exchange factors for ARF family members (see below). These results also suggest a possible link between integrin activation, GTP-loading of ARF family members, and PI(3)K. Previously reported effects of PI(3)K inhibitors on integrin activation, cell adhesion and cell migration could be explained by a model in which PtdIns(3,4,5)P₃ recruits cytohesins to the membrane (Fig. 2). However, there is as yet no evidence that PI(3)K products affect the function of these proteins *in vivo*.

PTB domains. Structural studies have shown that phosphotyrosine-binding domains (PTB domains) represent a subgroup of PH domains that have binding sites for phosphotyrosine proteins. One of these proteins, the Shc PTB domain, has been shown to bind to PtdIns(4,5)P₂ in competition with phosphopeptides²¹. The Shc PTB domain has also been shown to bind PtdIns(3,4,5)P₃ with similar affinity to PtdIns(4,5)P₂ (ref. 22). Because PtdIns(4,5)P₂ is much more abundant than PtdIns(3,4,5)P₃, it is unlikely that PtdIns(3,4,5)P₃ binding to the Shc PTB domain is physiologically relevant. However, the competition between phosphoinositide binding and phosphotyrosine peptide binding to the Shc PTB domain is analogous to the results observed for SH2 domains⁷. Thus these two unrelated structures will both bind phosphoproteins in competition with phosphoinositides, suggesting an important physiological function for this competition. Whether other PTB domains bind D3 phosphoinositides remains to be determined.

Activation of PKC by D3 phosphoinositides. Several studies have provided evidence that PKC is activated by PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ (refs 23–25). A family of related PKCs is subdivided according to cofactor requirements and dependence on lipid activators. Synthetic phosphoinositides, particularly PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, have been used by several laboratories to demonstrate activation of a number of PKC isoforms, particularly the novel PKC- ϵ , PKC- η (ref. 24) and atypical PKC- ζ (ref. 25), as well as the PKC-related kinase PRK1 (ref. 23). However, activation of PKCs by phosphoinositides *in vitro* is highly dependent on the assay conditions used, and ultimately, activation of PKC by PI(3)K products has to be determined *in vivo*. More recent studies have begun to address this question.

Both PKC- ϵ and PKC- λ are activated downstream of PI(3)K in stimulated cells^{26,27}. *In vitro*, the *Aplysia californica* PKC- ϵ homologue Apl II is also potentially activated by PtdIns(3,4,5)P₃, and *in vivo* activation of Apl II in response to insulin is inhibited by wortmannin²⁸. The phosphorylation of the PKC substrate pleckstrin can be inhibited by wortmannin in stimulated platelets and, in permeabilized cells, pleckstrin phosphorylation can be induced in the presence of synthetic PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, suggesting that a physiological substrate of PKC is downstream of PI(3)K²⁹. PtdIns(3,4,5)P₃ also binds with high affinity to a peptide derived from the PKC substrate neurogranin, suggesting that, as well as directly activating PKCs, PtdIns(3,4,5)P₃ may recruit and localize substrates at the plasma membrane, facilitating phosphorylation by PKCs³⁰. Finally, PtdIns(3,4,5)P₃ added exogenously to cells stimulates chemotaxis in a PKC-dependent manner by fusing with the plasma membrane³¹. These studies suggest that there are multiple redundant signals to activate PKCs *in vivo*.

The physiological relevance of activating PKC downstream of PI(3)K is unknown. One pathway that is likely to be affected by PKC is the Ras/Raf/MEK/MAP kinase pathway. Receptor tyrosine kinases activate Ras, which recruits Raf-1 to the plasma membrane, thereby initiating a kinase cascade that results in the activation of MAP kinase. Several studies have shown that wortmannin can block activation of Raf-1 and/or MAPK in some cell types³². Constitutively active mutants of p110- γ (ref. 33) but not p110- α (ref. 34), can activate MAPK in transfected cells. Because phorbol

esters can activate Raf-1 *in vivo*, and PKCs can phosphorylate Raf-1 *in vitro*, it could be argued that wortmannin is blocking PKC-dependent activation of Raf-1 (Fig. 2). Given the complexity of Raf-1 and MAPK regulation, it would not be surprising if the products of PI(3)K affect multiple steps in this pathway.

Binding of PtdIns(3)P to adaptin AP-2. Genetic studies from the yeast *Saccharomyces cerevisiae* have indicated a role for PI(3) kinases, particularly Vps34 and its product PtdIns(3)P, in vesicle trafficking³⁵. A mammalian homologue of the yeast Vps34 has been identified and, because these enzymes specifically produce PtdIns(3)P, a role for this lipid in vesicle trafficking in both yeast and mammalian cells is suggested.

Adapter protein-2 (AP-2) is the only protein with high affinity and isomer-specific binding to PtdIns(3)P so far identified³⁶. AP-2 is involved in assembling clathrin coats at the plasma membrane. The μ subunit of this protein specifically binds to the YXRL sequence motif found on TGN38 and certain other proteins that internalize through coated pits. Relatively low concentrations of PtdIns(3)P enhance binding to the YXRL peptide³⁶. Although PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ also enhance binding, they are less effective than PtdIns(3)P. This high specificity for PtdIns(3)P is likely to be physiologically relevant as other phosphoinositide-binding proteins seem to have very low affinity for this lipid.

These results have suggested a role for PtdIns(3)P in either the assembly or the loading of proteins into coated pits. Such a model might explain why Vps34 is required for trafficking of soluble hydrolases from the *trans*-Golgi network to the vacuole if PtdIns(3)P is required for loading or assembly of these vesicles. It remains to be seen whether PtdIns(3)P affects protein loading by adaptins other than AP-2 that function at the plasma membrane. In addition, recent studies indicate that the α -subunit of several adaptins binds with high specificity to PtdIns(3,4,5)P₃ and that this lipid disrupts binding to clathrin³⁷. Thus PtdIns(3)P and PtdIns(3,4,5)P₃ may have distinct roles in adaptin function.

In mammalian cells, the p85/p110 PI(3)K, which produces both PtdIns(3)P and PtdIns(3,4,5)P₃, is also implicated in vesicle trafficking. This enzyme is required for PDGF-dependent trafficking of the PDGF receptor to lysosomes³⁸ and for insulin-dependent trafficking of the glucose transporter GLUT4 to the plasma membrane³⁹. Although yeasts do not have the enzymes to produce PtdIns(3,4,5)P₃, this lipid may be used for regulated trafficking in higher eukaryotic cells. The possibility that PtdIns(3,4,5)P₃ acts through an ARNO/cytohesin-1/Grp1 family member to regulate assembly of coated vesicles is discussed above.

Binding of PtdIns(3,4,5)P₃ to synaptotagmin. D3 phosphoinositides may also play a role in exocytosis. Recently, the C2B domain of the synaptic vesicle membrane protein synaptotagmin was shown to bind with high affinity to PtdIns(4,5)P₂, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ (ref. 40). Increasing the calcium ion concentration to levels equal to those required for neurotransmitter release at nerve terminals switched the specificity of binding from PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂. A model is therefore proposed in which synaptotagmin bound to PtdIns(3,4,5)P₃ in synaptic vesicles could sense a rise in cytosolic calcium⁴⁰. This would mobilize the vesicles to fuse with the presynaptic membrane, where the high calcium concentration would switch binding of PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂. There is a wealth of evidence implicating calcium and phosphoinositides in exocytosis; these results provide the first direct evidence for a mechanism by which an increase in the free calcium concentration and synthesis of D3 phosphoinositides regulate exocytosis.

Other targets of D3 phosphoinositides. The availability of synthetic analogues of D3 phosphoinositides has led to the discovery of a number of other proteins that bind with high affinity to PtdIns(3)P, PtdIns(3,4)P₂ or PtdIns(3,4,5)P₃. Centaurin- α was recently cloned in a screen for Ins(1,3,4,5)P₄-binding, which could be competed with PtdIns(3,4,5)P₃ (ref. 41). Centaurin- α

shows homology to ARF GAP, suggesting that it may be involved in ARF-dependent vesicle traffic.

D3 phosphoinositides also bind to a number of proteins that regulate actin assembly. The actin-capping protein profilin binds PtdIns(3,4)P₂ with higher affinity than PtdIns(3,4,5)P₃ and PtdIns(4,5)P₂ (ref. 42). Consistent with these relative binding affinities, PtdIns(3,4)P₂ binding also inhibits the ability of profilin to suppress actin polymerization as a result of formation of a complex between actin and profilin *in vitro*. Similarly, PtdIns(3,4)P₂ is more effective than PtdIns(4,5)P₂ at inactivating the actin-severing activity of gelsolin⁴³, and addition of PtdIns(3,4)P₂ or PtdIns(3,4,5)P₃ to permeabilized platelets results in uncapping of actin filaments⁴⁴. However, the relatively low amounts of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ compared to PtdIns(4,5)P₂ in stimulated cells suggests that D3 phosphoinositides do not play a direct role in the regulation of actin assembly, except in a few specific cases⁴³. In most cases, PtdIns(4,5)P₂ is the relevant phosphoinositide responsible for mediating actin assembly⁴⁴.

Wortmannin has been shown to inhibit PDGF-stimulated membrane ruffling in fibroblasts⁴⁵. PDGF can stimulate GDP/GTP exchange on Rac by a PI(3)K-dependent mechanism, suggesting that PI(3)K is upstream of Rac⁴⁶. Consistent with this idea, activated mutants of PI(3)K induce rearrangement of actin filaments similar to that observed in response to activated Rac⁴⁷. The mechanism by which D3 phosphoinositides signal to Rac is unknown, but may involve binding and/or stimulation of a PH-domain-containing exchange factor for Rac (Fig. 2).

Conclusions and perspectives

Phosphatidylinositol is unique among the lipids in that it can undergo reversible phosphorylation at multiple sites to generate five different phosphoinositides. Because it is not generally found in prokaryotic cells, PtdIns is likely to have evolved for the purpose of the more complex regulation needed in membranes of multicompartment eukaryotic cells. Similarly, lower eukaryotes such as yeast produce only PtdIns(3)P, and it is likely that PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ have evolved to regulate more complex signal transduction mechanisms in higher eukaryotes. Understanding the role of these phosphoinositides in intracellular signalling is complicated by the evidence that multiple proteins have independently evolved binding sites for specific phosphoinositides. Individual PH domains have evolved specificity for PtdIns(3,4)P₂, PtdIns(4,5)P₂ or PtdIns(3,4,5)P₃, whereas the SH2 domains examined so far can only bind with high affinity to PtdIns(3,4,5)P₃. The adaptin AP-2 is the only protein that has been shown to interact specifically with PtdIns(3)P. It is likely that there are many other proteins with activities and cellular localizations that are regulated by binding to D3 phosphoinositides. The model that emerges is that local production of these lipids aids in the recruitment of subgroups of signalling proteins for efficient cellular responses to extracellular stimuli (Fig. 2). The availability of cDNA clones for many of the PI(3)Ks, cDNA clones of several putative intracellular targets of the phosphoinositides, and synthetic forms of the phosphoinositides, should accelerate progress in understanding the importance of these lipids in cellular regulation. □

Alex Toker is at the Boston Biomedical Research Institute, 20 Staniford Street, Boston, Massachusetts 02114, USA (e-mail: toker@bbri.harvard.edu); Lewis Cantley is in Department of Cell Biology, Harvard Medical School and Division of Signal Transduction, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, Massachusetts 02115, USA (e-mail: cantley@helix.mgh.harvard.edu).

- Whitman, M., Downes, C. P., Keeler, M., Keller, T. & Cantley, L. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* **332**, 644–646 (1988).
- Traynor-Kaplan, A. E., Harris, A. L., Thompson, B. L., Taylor, P. & Sklar, L. A. An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature* **334**, 353–356 (1988).
- Frank, T. F. et al. PI3K: downstream AKTion blocks apoptosis. *Cell* **88**, 435–437 (1997).
- Chang, H. W. et al. The retroviral oncogene p3k is homologous to the gene encoding for the catalytic subunit of phosphoinositide 3-kinase. *Science* (in the press).
- Morris, J. Z., Tissenbaum, H. A. & Ruvkun, G. A phosphatidylinositol-3-OH kinase family member

- regulating longevity and diapause in *Caenorhabditis elegans*. *Nature* **382**, 536–539 (1996).
- Rodriguez, V. P. et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370**, 527–532 (1994).
- Rameh, L., Chen, C.-S. & Cantley, L. C. Phosphatidylinositol-3,4,5-P₃ interacts with SH2 domains and modulates phosphoinositide 3-kinase association with tyrosine-phosphorylated proteins. *Cell* **83**, 821–830 (1995).
- Frank, T. F. et al. The protein kinase encoded the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**, 1–20 (1995).
- Burgering, B. M. & Coffey, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599–602 (1995).
- Kohn, A. D., Takeuchi, F. & Roth, R. A. Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J. Biol. Chem.* **271**, 21920–21926 (1996).
- Frank, T. F., Kaplan, D. R., Cantley, L. C. & Tokier, A. Direct regulation of the Akt protooncogene product by PI34P2. *Science* **275**, 665–668 (1997).
- Klippel, A., Kavanaugh, W. M., Pot, D. & Williams, L. T. A specific product of PI 3-K directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol. Cell. Biol.* **17**, 338–344 (1997).
- Frech, M. et al. High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J. Biol. Chem.* **272**, 8474–8481 (1997).
- Alessi, D. R. et al. Characterisation of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates PKB-α. *Curr. Biol.* **7**, 261–269 (1997).
- Konishi, H. et al. Activation of RAC-protein kinase by heat shock and hyperosmolarity stress through a pathway independent of PI 3-K. *Proc. Natl Acad. Sci. U.S.A.* **93**, 7639–7643 (1996).
- Salim, K. et al. Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Btk. *EMBO J.* **15**, 6241–6250 (1996).
- Fukuda, M., Kojima, T., Kabayama, H. & Mikoshiba, K. Mutation of the pleckstrin homology domain of BTK in immunodeficiency impaired IP4 binding capacity. *J. Biol. Chem.* **271**, 30303–30306 (1996).
- Klarulund, J. K. et al. Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 Homology domains. *Science* **275**, 1927–1930 (1997).
- Kolanus, W. et al. Alpha L beta 2 integrin/LFA-1 binding to ICAM-1 induced by cytohesin-1, a cytoplasmic regulatory molecule. *Cell* **86**, 233–242 (1996).
- Chardin, P. et al. A human exchange factor for ARF contains Sec7 and PH domains. *Nature* **384**, 481–484 (1996).
- Zhou, M.-M. et al. Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature* **378**, 584–592 (1995).
- Rameh, L. E. et al. Phosphoinositide binding specificity of pleckstrin homology domains; the Btk domain specifically binds to PIP3. *J. Biol. Chem.* (in the press).
- Palmer, R. H. et al. Activation of PRK1 by phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **270**, 22412–22416 (1995).
- Toker, A. et al. Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃. *J. Biol. Chem.* **269**, 32358–32367 (1994).
- Nakanishi, H., Brewer, K. A. & Exton, J. H. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **268**, 13–16 (1993).
- Moriya, S. et al. Platelet-derived growth factor activates protein kinase C-ε through redundant and independent signaling pathways involving phospholipase C-γ or phosphatidylinositol 3-kinase. *Proc. Natl Acad. Sci. USA* **93**, 151–155 (1996).
- Akimoto, K. et al. EGF or PDGF receptors activate atypical PKCA through phosphatidylinositol 3-kinase. *EMBO J.* **15**, 788–798 (1996).
- Sossin, W. S., Chen, C.-S. & Tokier, A. Stimulation of an insulin receptor activates and downregulates the Ca-independent protein kinase C, Apl II, through a wortmannin sensitive signaling pathway in Aplysia. *J. Neurochem.* **67**, 220–228 (1996).
- Toker, A. et al. Phosphorylation of the platelet p47 phosphoprotein is mediated by the lipid products of phosphoinositide 3-kinase. *J. Biol. Chem.* **270**, 29525–29531 (1995).
- Lu, P. J. & Chen, C.-S. Selective recognition of PIP3 by a synthetic peptide. *J. Biol. Chem.* **272**, 466–472 (1997).
- Derman, M. P. et al. The lipid products of phosphoinositide 3-kinase mediate chemotaxis through protein kinase. *J. Biol. Chem.* **272**, 6465–6470 (1995).
- Cross, D. A. et al. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem. J.* **303**, 21–26 (1994).
- Lopez-Illasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S. & Wetzker, R. Linkage of G protein coupled receptors to the MAPK signaling pathway through PI 3-K. *Science* **275**, 394–397 (1997).
- Klippel, A. et al. Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathway. *Mol. Cell. Biol.* **16**, 4117–4127 (1996).
- Schu, P. V. et al. Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science* **260**, 88–91 (1993).
- Rapaport, I. et al. Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes. *EMBO J.* **16**, 2240–2250 (1997).
- Hao, W. et al. Regulation of AP-3 function by inositides: identification of PIP3 as a potent ligand. *J. Biol. Chem.* **272**, 6393–6398 (1997).
- Joly, M., Kazlauskas, A., Fay, F. S. & Corvera, S. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. *Science* **263**, 684–687 (1994).
- Frevert, E. U. & Kahn, B. B. Differential effects of constitutively active PI 3-K on glucose transport, glycogen synthesis activity and DNA synthesis in 3T3-L1 adipocytes. *Mol. Cell. Biol.* **17**, 190–198 (1997).
- Schiavo, G., Gu, Q.-M., Prestwich, G. D., Sollner, T. H. & Rothman, J. E. Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin. *Proc. Natl Acad. Sci. USA* **93**, 13327–13332 (1996).
- Hammonds-Odie, L. P. et al. Identification and cloning of centaurin-α. *J. Biol. Chem.* **271**, 18859–18868 (1996).
- Lu, P. J., Shieh, W.-R., Rhee, S. G., Yin, H. L. & Chen, C.-C. Lipid products of PI 3-K bind profilin with high affinity. *Biochemistry* **35**, 14027–14034 (1997).
- Hartwig, J. H. et al. D3 phosphoinositides and outside-in integrin signaling by GPIIb/IIIa mediate platelet actin assembly and filipodial extension induced by PMA. *J. Biol. Chem.* **271**, 32986–32993 (1996).
- Hartwig, J. H. et al. Thrombin receptor-ligation and activated Rac uncouple actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* **82**, 1–20 (1995).
- Wennstrom, S. et al. Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol.* **4**, 385–393 (1994).
- Hawkins, P. T. et al. PDGF stimulates an increase in GTP-rac via activation of phosphoinositide 3-kinase. *Curr. Biol.* **5**, 393–403 (1995).
- Reif, K., Nobes, C. D., Thomas, G., Hall, A. & Cantrell, D. A. PI 3-K signals activate a selective subset of Rac/Rho-dependent effector pathways. *Curr. Biol.* **6**, 1445–1455 (1996).

The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function

Joseph Torchia*, David W. Rose†, Juan Inostroza*, Yasutomi Kamei*, Stefan Westin‡, Christopher K. Glass‡ & Michael G. Rosenfeld*

* Howard Hughes Medical Institute, † UCSD Department of Medicine and Whittier Diabetes Program, ‡ Cellular and Molecular Medicine, School and Department of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0648, USA

The functionally conserved proteins CBP and p300 act in conjunction with other factors to activate transcription of DNA. A new factor, p/CIP, has been discovered that is present in the cell as a complex with CBP and is required for transcriptional activity of nuclear receptors and other CBP/p300-dependent transcription factors. The highly related nuclear-receptor co-activator protein NCoA-1 is also specifically required for ligand-dependent activation of genes by nuclear receptors. p/CIP, NCoA-1 and CBP all contain related leucine-rich charged helical interaction motifs that are required for receptor-specific mechanisms of gene activation, and allow the selective inhibition of distinct signal-transduction pathways.

CBP and p300 are functionally conserved proteins that have intrinsic acetylase activity^{1,2} and are essential for the activation of transcription by a large number of regulated transcription factors, including nuclear receptors (refs 3–6), CREB (refs 7, 8), AP-1 (ref. 8), bHLH factors (ref. 9) and STATs (refs 10–12). Nuclear receptors are a large family of ligand-dependent transcription factors that bind as homodimers or heterodimers to their cognate DNA elements^{13–15} and regulate genes involved in critical aspects of cell proliferation, differentiation and homeostasis. Transcriptional regulation by nuclear receptors depends primarily upon a ligand-dependent activation function, AF-2, located in the carboxy terminus and predicted to undergo an allosteric change upon ligand binding^{16–22}, although additional amino-terminal activation functions operate for many receptors. Consistent with this, CBP and p300 have been found to interact directly with nuclear receptors in a ligand- and AF-2-dependent manner.

In addition to CBP and p300, a series of factors that exhibit ligand- and AF-2-dependent binding to nuclear receptor C termini have been identified biochemically^{3,23–25} and by expression cloning^{3–6,27–32}. Two homologous factors, SRC-1/NCoA-1 and TIF-2/GRIP-1, which increase ligand-dependent transcription by several nuclear receptors in co-transfection assays^{1,30,31}, constitute a nuclear receptor co-activator (NCoA) gene family^{1,30,31}. These findings have raised intriguing questions of whether NCoA-1, CBP or other p160 family members are required for ligand-dependent gene activation.

Here we report the cloning and characterization of a new NCoA/SRC family member, p/CIP, which complexes with a significant portion of CBP in the cell. Surprisingly, both p/CIP and NCoA-1 are required for the function of nuclear receptors, whereas p/CIP, but not NCoA-1, is required for the function of other CBP-dependent transcription factors. A series of helical leucine-charged residue-rich domains (LCDs) serve as interaction motifs within these factors and are required for the assembly of a co-activator complex and provide specificity of nuclear-receptor activation.

New members of the NCoA family

Our initial expression screening strategy for identifying members of the p160 gene family was based on the observation that the biochemically identified p160 proteins interact with a 100-amino-acid region in the C terminus of CBP (residues 2,058–2,170), as well

as with the liganded oestrogen receptor¹. This enabled us to isolate previously described NCoA-1/SRC-1 protein and a second related factor, NCoA-2 (Fig. 1a), which has a relative molecular mass of 159.6K and seems to be the murine homologue of human TIF-2 (ref. 30), part of which has been reported as GRIP-1 (ref. 31). In addition, we identified a related factor which we term p/CIP (for p300/CBP/co-integrator-associated protein) (Fig. 1a).

p/CIP is a 152K protein which is highly related to SRC-1/NCoA-1 and NCoA-2/TIF-2, having an overall amino-acid identity of 31% and 36%, respectively (Fig. 1d). p/CIP harbours a fairly well conserved N-terminal bHLH, PAS A domain (50–60% amino-acid identity), a serine/threonine-rich region, and a C-terminal glutamine-rich region, which are also present in NCoA-1 and NCoA-2. Western blot analysis indicates that p/CIP, NCoA-1 and NCoA-2 are widely expressed in adult tissues and in all cell lines tested (Fig. 1b,c).

A CBP/pCIP complex

To evaluate the association of p/CIP, NCoA-1 and NCoA-2 with CBP and nuclear receptors, a glutathione S-transferase fusion protein (GST) with CBP (residues 2,058–2,170) was used to affinity-purify interacting proteins from HeLa cell extract. p/CIP was consistently observed by immunoblotting using affinity-purified anti-p/CIP IgG, whereas much smaller amounts of NCoA-1 were detected by immunoblotting with anti-NCoA-1 IgG (Fig. 2a). Similarly, immunoprecipitations from whole-cell extracts using excess antiserum selective for each protein, followed by immunoblotting with anti-CBP/p300 antibody demonstrated that the vast majority of CBP/p300 co-precipitated with the new factor p/CIP, although small amounts of NCoA-1- and NCoA-2-associated CBP could be detected (Fig. 2b). Conversely, the amount of CBP/p300 remaining in the supernatant fraction following immunodepletion with anti-NCoA-1 IgG remained unchanged, but a significant fraction of CBP/p300 CBP was removed by immunodepletion with anti-p/CIP IgG (Fig. 2b). These results indicated that p/CIP could form a complex with p300/CBP in the cell.

To define the CBP-interaction domain in p/CIP, we generated deletion mutants and tested against CBP (amino acids, 2,058–2,170) by a yeast two-hybrid assay. The major CBP interaction

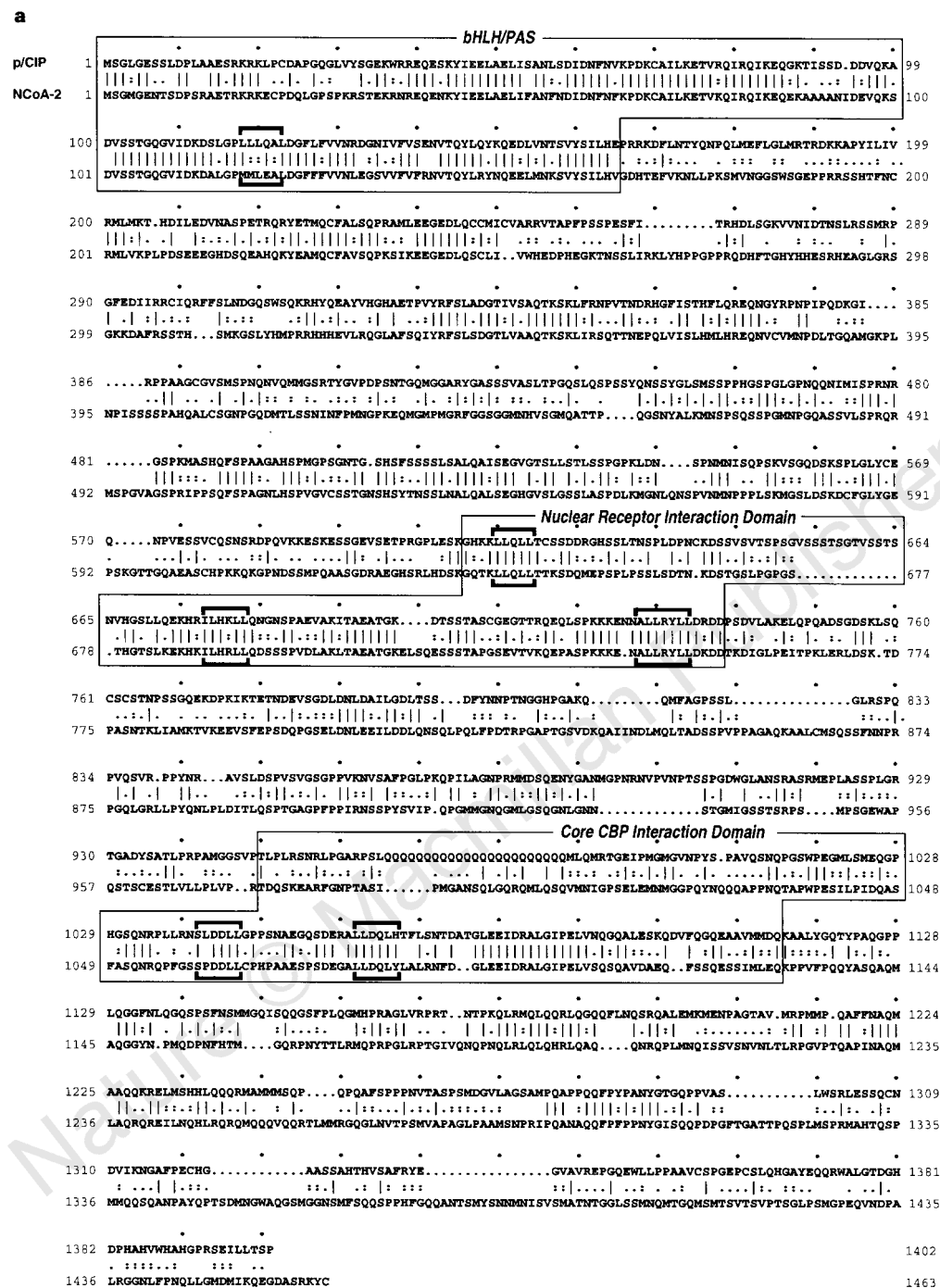
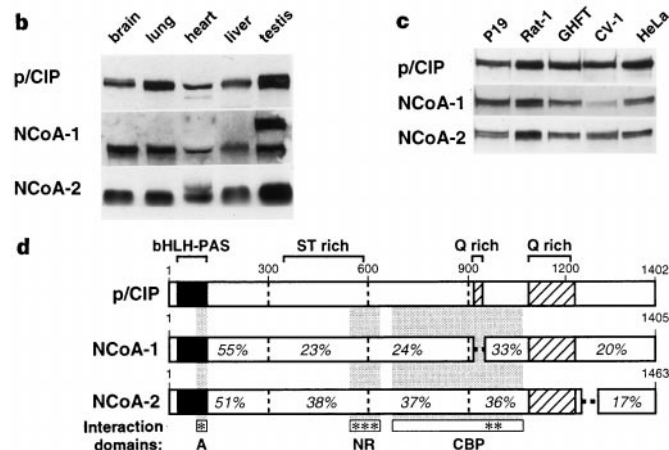


Figure 1 Characterization of a CBP-associated factor (p/CIP) and a related member of the NCoA family (NCoA-2). **a**, Comparison of the primary amino-acid sequences of p/CIP and NCoA-2. The conserved bHLH, PAS A domain, the nuclear-receptor-interaction domains and the minimal nuclear-receptor and CBP interaction domains are boxed, and repeat motifs involved in critical interactions are bracketed. **b**, **c**, Western blot analysis of total cell extracts for p/CIP, NCoA-1 and NCoA-2 in various tissues and cell lines; there is widespread expression of all three proteins, but relative levels differ. **d**, Diagram of p/CIP, showing regions of homology with NCoA-1 and NCoA-2. Asterisks refer to the repeated peptide motifs that appear to be functionally important (Figs 5, 6).



domain was located between residues 758 and 1,115, with an internal 200-amino-acid domain that could still interact. We observed a less pronounced interaction with the N-terminal region containing the PAS A domain (Fig. 2c). A single nuclear-receptor-interaction domain (residues 591–803) was localized N-terminal of the CBP/p300 interaction domain (Fig. 2c). Further mapping delineated a minimal nuclear-receptor-interaction region encompassing amino acids 680–740 in p/CIP which were sufficient for binding to the liganded nuclear receptors (data not shown). Comparable regions in NCoA-1 and NCoA-2 were found to mediate interactions with both CBP/p300 and nuclear receptors (Fig. 2d, and data not shown). GST pull-down assays of whole-cell extracts revealed that p/CIP, NCoA-1 and NCoA-2 interacted with GST fusion proteins with the oestrogen receptor (GST-ER) and the retinoic acid receptor (GST-RAR) in a ligand-dependent manner (Fig. 2e).

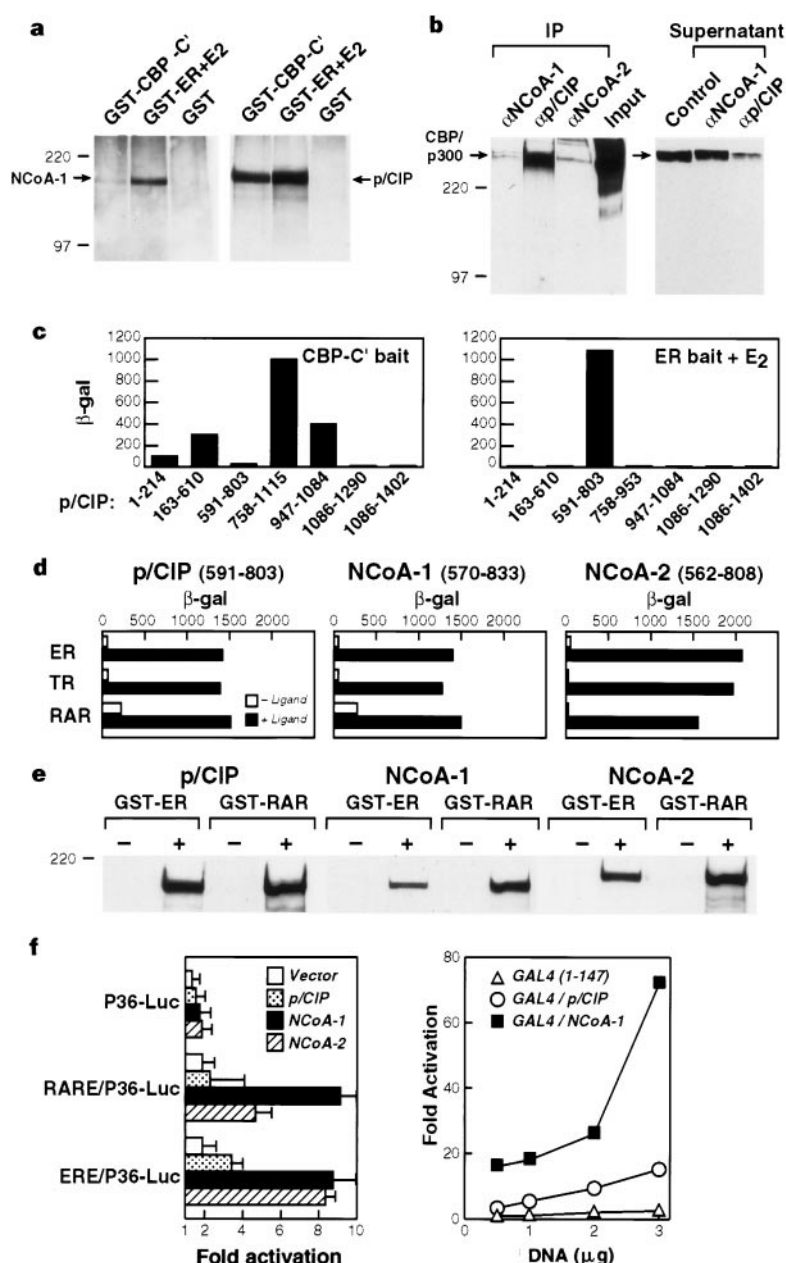


Figure 2 Biochemical analysis of p/CIP and NCoA factors. **a**, Interaction between recombinant GST proteins and NCoAs from HeLa whole-cell extracts detected using an antibody against p/CIP (left) or NCoA-1 (right). **b**, Left, co-immunoprecipitation of CBP/p300 and p/CIP. Anti-p/CIP, NCoA-1 or NCoA-2 IgG were incubated with HeLa whole-cell extracts and immunocomplexes were separated by SDS-PAGE and probed using anti-CBP/p300 IgG; right, detection of CBP/p300 in supernatant following immunodepletion of whole-cell extracts with specific anti-NCoA antibodies. **c**, Yeast two-hybrid assay mapping regions of interaction between p/CIP and CBP C terminus (amino acids 2,058–2,170) and liganded nuclear receptors (LBD). **d**, A common nuclear-receptor-interaction domain is found in p/CIP, NCoA-1 and NCoA-2 by yeast two-hybrid assays. Ligands (+) were oestradiol (10^{-6} M), Triac (10^{-6} M) and retinoic acid (10^{-6} M). **e**, p/CIP, NCoA-1 and NCoA-2 interactions with nuclear receptors *in vitro*. Recombinant GST-nuclear-receptor proteins were incubated with whole-cell extract in the presence (+) or absence (–) of ligand, and western-blotted using p/CIP-, NCoA-1- or NCoA-2-specific IgG. **f**, Reporters containing the minimal prolactin promoter (P-36 luciferase) alone or 2 copies of the indicated response elements and expression plasmids expressing p/CIP, NCoA-1 or NCoA-2 were transfected into HeLa cells in the presence of the corresponding ligand. Right, effects of varying amounts of plasmid expressing GAL4(1–147), GAL4-NCoA-1 or GAL4-p/CIP fusion proteins on a minimal (UAS)₆-dependent reporter.

Co-transfection with NCoA-1/SRC-1 or NCoA-2/TIF-2 expression vectors potentiated ligand-dependent activation (generally three- to eightfold), whereas co-transfection with p/CIP expression plasmids resulted in minimal or no activation effect (Fig. 2f, left). In addition, when full-length cDNAs were fused to GAL4(1–147), the activation observed by GAL–NCoA-1 was significantly stronger than GAL–p/CIP (Fig. 2f, right). Co-transfection of CBP and NCoA-1 or NCoA-2 expression vectors resulted in variable synergy (data not shown), consistent with findings reported for SRC-1 (ref. 33).

Nuclear receptors require p/CIP and NCoA-1

To investigate the function of p/CIP, NCoA-1 and NCoA-2, we used microinjection techniques with affinity-purified IgGs. Reporter genes were placed under the control of a minimal promoter containing either nuclear receptor or other response elements, as described³. Microinjection of anti-p/CIP IgG prevented retinoic acid from activating a retinoic-acid receptor (RAR)-dependent transcription unit (Fig. 3a), but had no effect on a promoter under the control of SP-1 elements or the cytomegalovirus (CMV) promoter. In similar experiments, we found that p/CIP

was also required for the actions of oestrogen, thyroid-hormone and progesterone receptors (Fig. 3b).

To determine whether depletion of CBP, rather than p/CIP itself, was responsible for the observed effects, we next evaluated the relative abilities of p/CIP, CBP, NCoA-1 and/or NCoA-2 to rescue the inhibitory effect of anti-p/CIP IgG. No factor alone, including CBP, was able to rescue the inhibition by anti-p/CIP IgG of RAR-dependent transcription, indicating that steric blockage or removal of CBP did not account for the observed effects. However, the simultaneous expression of both p/CIP and CBP fully restored the retinoic-acid transcriptional response in anti-p/CIP-treated cells (Fig. 3c). Therefore CBP and p/CIP are required together for nuclear-receptor activation. To confirm this strict requirement for p/CIP, the action of a 137-amino-acid region of p/CIP (residues 947–1084) containing the core CBP-interaction domain was tested by microinjection assay, resulting in complete inhibition of the retinoic-acid-dependent gene activation (Fig. 3d, left). In contrast, this fragment did not block the activity of non-CBP-dependent promoters (Fig. 3d, right).

We next investigated whether p/CIP might also be required for

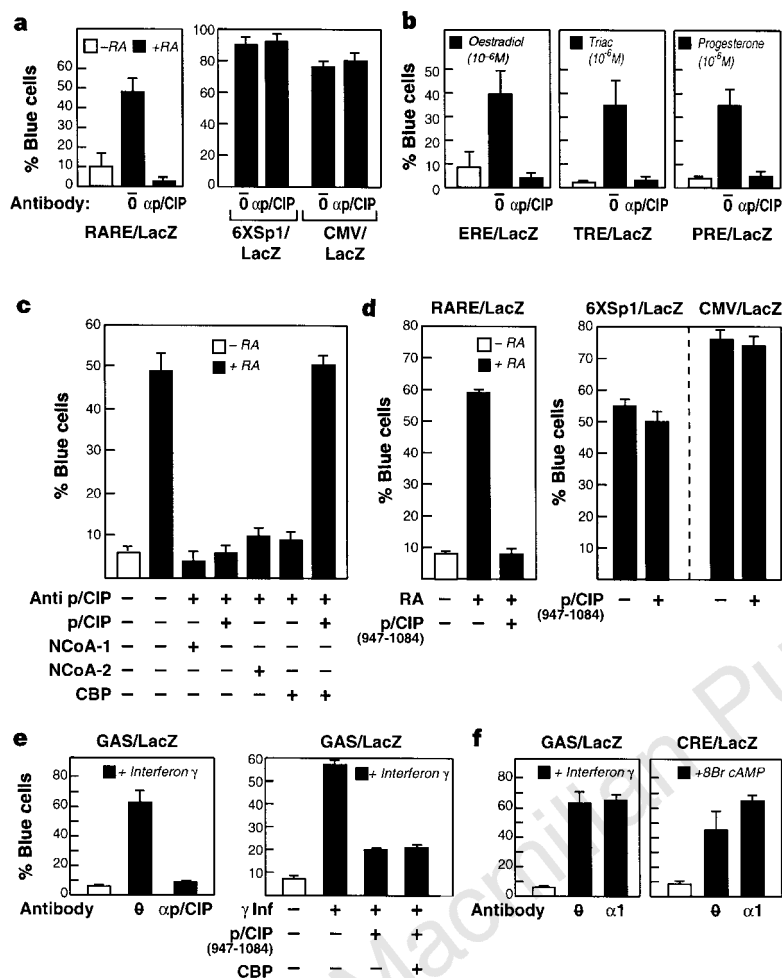


Figure 3 Role of P/CIP in function of CBP-dependent transcription factors. **a**, Effects of microinjection in Rat-1 cells of affinity-purified anti-p/CIP IgG on ligand-dependent gene activation by RAR. **b**, Similar experiments with minimal promoters with four copies of the oestrogen- (ERE), thyroid-hormone (TRE) or progesterone (PRE)-receptor response elements. **c**, Requirement for both CBP and p/CIP expression vectors to rescue anti-p/CIP IgG inhibition of RAR-dependent gene activation. **d**, Effects of expression of the p/CIP core CBP interaction domain (947–1,084) on RAR (left), and SP-1 or CMV(right)-dependent transcription. **e**, Effects of anti-p/CIP IgG (α p/CIP) on an IFN γ -dependent promoter (GAS/LacZ)¹² (left). Effects of p/CIP (residues 947–1,084) on IFN γ -stimulated transcriptions and failure of CBP expression vector to rescue this inhibition (right). **f**, Effects of anti-NCrA-1 IgG (α 1) on GAS and cAMP-dependent (2 $\times$ CRE) promoters. Experiments were done at least three times, with >200 cells injected; error bars are $\pm 2 \times$ s.e.m.

transcriptional activation by other CBP-dependent transcription factors, such as STATs¹⁰⁻¹². Therefore the effects of anti-p/CIP and NCoA-1 IgG were evaluated by immunoinjection assay in cells, initially using interferon (IFN)- γ -dependent reporters or reporters dependent on TPA (12-O-tetradecanoylphorbol-13-acetate). Anti-p/CIP IgG entirely inhibited STAT-dependent and TPA-dependent transcriptional activation events (Fig. 3e, and data not shown), which was not restored by overexpression of CBP alone (data not shown). Independent confirmation was provided by overexpression of the CBP-interaction domain of p/CIP (residues 947-1,084), which blocked the stimulation of transcription by interferon- γ or TPA (Fig. 3e, and data not shown). C-terminally truncated CBP failed to enhance either IFN- γ - or TPA-dependent transcription in co-transfection assays (data not shown), and could not rescue the block of retinoic-acid and IFN γ -dependent gene activation by injected anti-CBP IgG^{3,12} (data not shown). These results indicate that p/CIP and CBP are a functional complex, necessary for the activity of several CBP-dependent transcription factors as well as nuclear receptors.

Roles of NCoA-1 and NCoA-2

As p/CIP is required by nuclear receptors and several CBP/p300-dependent transcription factors, we evaluated the roles of NCoA-1/SRC-1 and NCoA-2/TIF-2, which by co-transfection enhance transactivation by nuclear receptors. Microinjection of anti-NCoA-1 IgG, but not anti-NCoA-2 IgG, effectively inhibited retinoic-acid-dependent transcription (Fig. 4a), whereas these antisera failed to inhibit several control promoters lacking nuclear-receptor response elements (Fig. 4a). In addition, anti-NCoA-1 IgG fully inhibited oestrogen- and thyroid hormone-receptor stimula-

tion (Fig. 4b) and partly inhibited progesterone-receptor stimulation (Fig. 4b). Co-injection of NCoA-1, NCoA-2 or p/CIP expression vectors revealed that the inhibitory effects of anti-NCoA-1 IgG could be reversed by either NCoA-1 or NCoA-2, but not by p/CIP (Fig. 4c), consistent with a distinct role for this factor, and in contrast to the requirement for both p/CIP and CBP to rescue inhibition by anti-p/CIP IgG. Co-injection of a CMV-CBP expression vector also failed to restore activity, consistent with the idea that both NCoA-1 and the CBP/p300/p/CIP complex are independently required for nuclear-receptor gene activation (Fig. 4c). In contrast, anti-NCoA-1 IgG exerted no effects on either cAMP- or IFN γ -dependent reporters (Fig. 3f). These observations suggest that NCoA-1 is selectively required as a co-activator for the ligand-activated nuclear receptor gene expression events, while the requirement for the p300/CBP/p/CIP complex seems to reflect a more general obligatory role in gene activation events.

Inhibition by motifs of the p/CIP complex

Because of the striking relatedness of NCoA-1 and p/CIP, despite their different function, and the activation by CBP/p300 of different classes of transcription factors, we investigated whether distinct interaction domains could selectively block the actions of specific signal-transduction pathways in the nucleus. The nuclear-receptor interaction domains of p/CIP, NCoA-1 and NCoA-2 have revealed the presence of highly conserved LCDs that share a consensus core LXXLL sequence motif (Fig. 5a). This motif is found in both the nuclear-receptor and p/CIP-interaction domains of CBP, and in the CBP-interaction domain of p/CIP. Analysis of these interaction regions by the self-optimized prediction method³⁴ indicates that they are helical domains, generally with amphipathic characteristics

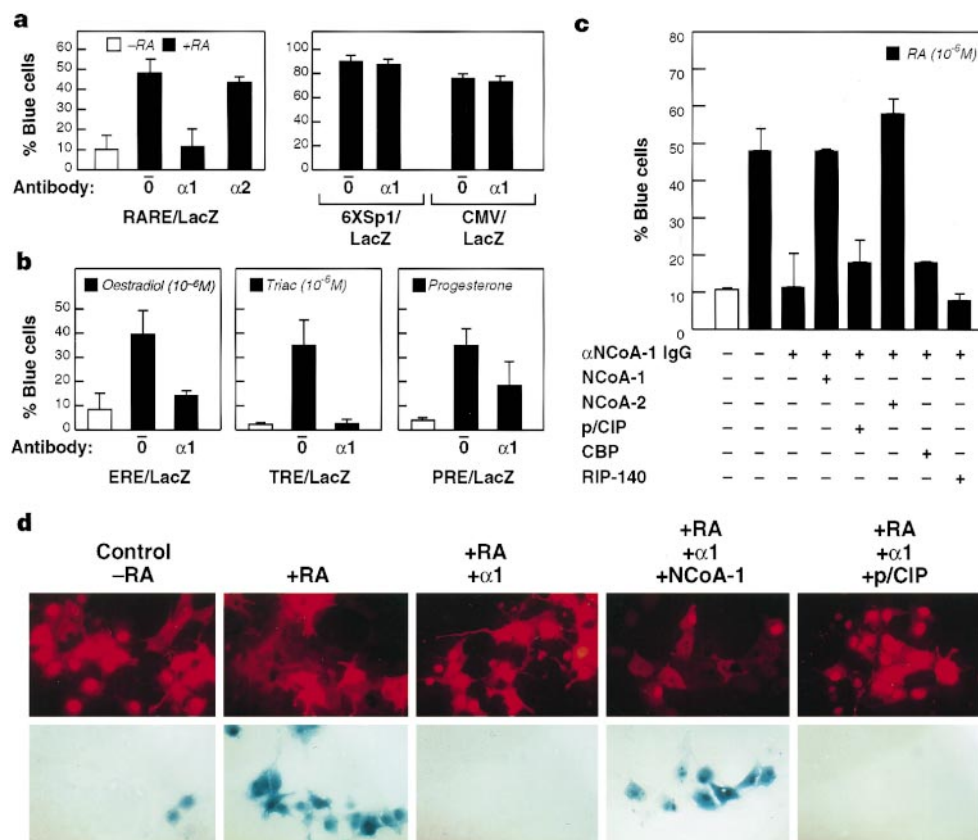


Figure 4 Role of NCoA-1 and NCoA-2 in nuclear receptor function. **a**, Microinjection of affinity-purified anti-NCoA-1, but not anti-NCoA-2 IgG blocked ligand-dependent gene activation by RAR (left) and did not inhibit expression of either the 6 × SP-1 or CMV-driven promoters (right). **b**, Results were similar using minimal promoters with two copies of the oestrogen (ERE) or T3R (TRE) response elements, with effects being less profound on progesterone(PRE)-mediated transcription. **c**, Anti-NCoA-1 IgG blocked retinoic-acid-dependent activation of the RARE/LacZ reporter, and was not rescued by CMV expression vectors expressing p/CIP or CBP; expression was fully rescued by either CMVNCOA-1 or CMVNCOA-2. **d**, Photomicrographs of rhodamine-stained injected cells and the corresponding protein of X-gal staining.

(Fig. 5a). We tested these LCDs for a critical interaction function by introducing four amino-acid mutations of this motif into the N terminus of CBP (residues 65–76), which abolishes interactions with nuclear receptors (Fig. 5b). The minimal nuclear-receptor-interaction domain of NCoA-1 contains three such helical domains, and a fourth domain (LCD6) is also present in a variant of NCoA-1 (refs 3, 29). To assess the importance of these domains in NCoA-1, a smaller region lacking helical domain 3 gave little or no decrease in binding to either oestrogen or retinoic-acid receptors; deletion of helical domain 1 gave a small but significant decrease (Fig. 5c). In contrast, a four-amino-acid substitution in the second NCoA-1 helical domain (LCD2, HRLL → AAAA), which alters the properties of this helix, abolished interaction with both oestrogen and retinoic-acid receptors. Conversely, a 37-amino-acid region of NCoA-1 containing LCD2, or a 59-amino-acid region containing LCD6, was sufficient for binding to liganded nuclear receptors (Fig. 5c, left). An excess of 24-amino-acid oligopeptide encompassing LCD2 effectively blocked interaction between liganded RAR and NCoA-1 *in vitro*, but a peptide corresponding to LCD1 was less effective. These results show that specific motifs can be both necessary and, in certain instances, sufficient for interaction.

To test for selective functional requirements of these helical motifs in the nuclear-receptor-interaction domain of NCoA-1, we generated mutations in helical domains 2 or 3 in the holoprotein, and determined whether they could rescue the anti-NCoA-1 IgG inhibition of retinoic-acid receptor. Wild-type NCoA-1 rescued activation, but a NCoA-1 holoprotein with clustered point mutations in helical domain 3 (LCD3-mut) was unable to rescue retinoic-acid receptor function. NCoA-1 containing a helical domain 2 (LCD2-mut) mutation retained some effect (Fig. 5d), consistent with the residual ability of the helical domains to mediate nuclear-receptor interactions. Surprisingly, LCD3-mut was fully functional in oestrogen-receptor-dependent gene activation, whereas LCD2-mut was now ineffective at rescuing oestrogen-receptor function (Fig. 5e). These results suggest

that the helical interaction motifs of NCoA-1 afford a level of receptor specificity.

To evaluate the importance of these motifs, we tested the corresponding peptides for their ability to inhibit specific activation. NCoA-1 contains two additional related helical interaction motifs, and a peptide encompassing one of these (LCD4) can block nuclear-receptor transcription factor function and does not impair STAT function (Fig. 6a). Furthermore, a mutation in this motif impairs the function of this region in p/CIP (data not shown). Thus, specific signal transduction pathways could be selectively blocked by different helical interaction motifs.

We therefore investigated whether other motifs, not required for nuclear-receptor activation, might also be critical for co-activator function for other classes of CBP-dependent transcription factors, selectively blocking different signal-transduction pathways. Taking advantage of the fact that there is a critical STAT interaction domain within the first 100 amino acids of CBP¹², we determined whether a sequence of the CBP N-terminal 100 amino acids, distinct from the nuclear-receptor domain, might mediate interaction with STAT-1 and also be required for STAT function. We evaluated the effects of peptides corresponding to N-terminal regions of CBP on STAT-1 or retinoic-acid receptor function and found a synthetic peptide against the N-terminal 22 amino acids of CBP (CBP N'P1; Fig. 6b) that markedly inhibited IFN γ -dependent gene activation but had no effect on the retinoic-acid receptor. The identical peptide, from which the N-terminal seven amino acids (MAENLLY) were deleted, abolished this effect (CBP N'P2; Fig. 6b), suggesting that this sequence encompassed a motif required for STAT interaction and function. Our results support the functional significance of the STAT-1 interaction motif already identified in the CBP N terminus¹².

We also tested whether the CBP N-terminal peptide could selectively block the inhibitory effects of STAT-1 or retinoic-acid-receptor-dependent transcription by evaluating its effects on stimulation by IFN- γ and retinoic acid. The simultaneous addition

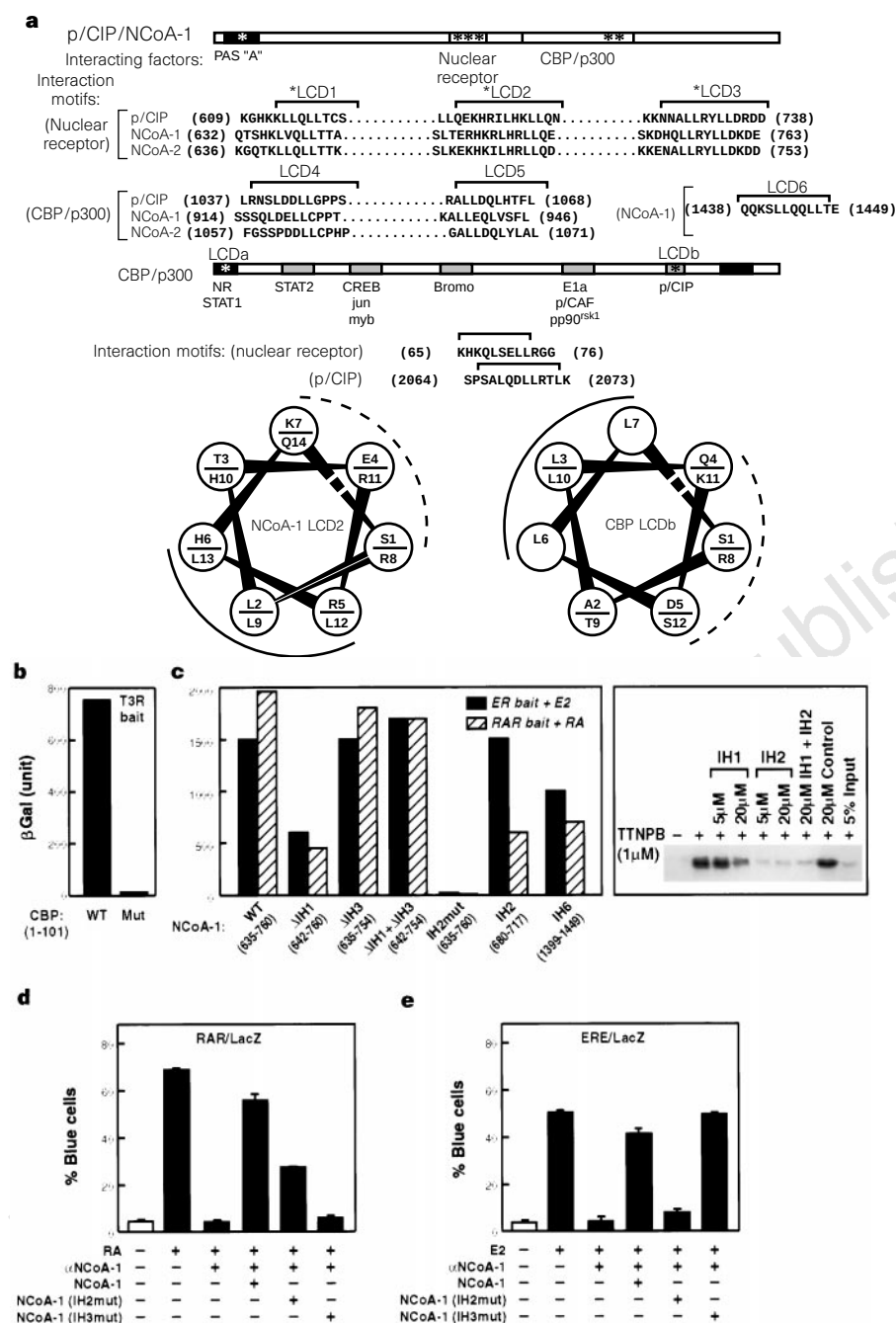


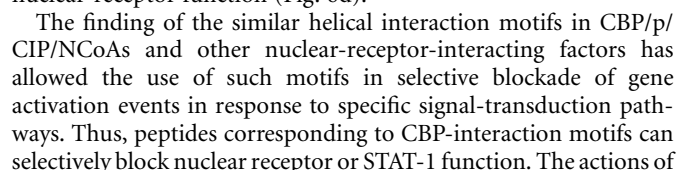
Figure 5 Leucine charged domains (LCDs) of p/CIP/NCOA-1/CBP. **a**, A repeated leucine-rich domain is required for protein-protein interactions between p/CIP, CBP, NCoAs and nuclear receptors. The sequences of some of these motifs are noted, with the core hexapeptide motifs indicated by brackets. Helical wheels of NCoA-1 LCD2 and CBP LCD6 are shown. **b**, Mutation of amino acids 70-73 in CBP (QLSELL → QLAAAA) resulted in a complete loss of ligand-dependent interaction with T3R. **c**, Left, assessment by the yeast two-hybrid assay of interactions between the NCoA-1 nuclear-receptor-interaction domains (residues 635-760) with nuclear receptors; centre, mutations of the LCD2 motif (RLHRL → RLAAAA) abolished ligand-dependent interaction, whereas peptides encompassing LCD2 (37 amino acids) alone or LCD6 (59 amino acids) were sufficient for ligand-dependent interaction; right, 24-mer peptides encompassing LCD1, LCD6, or a control peptide were tested for ability to inhibit binding of ³⁵S-labelled NCoA interaction domain fragment (amino acids 635-760) to liganded RAR with TTNPB (1 μM). **d**, **e**, Functional effects of plasmids expressing mutations in LCD2 (HRL → AAAA) and LCD3 (RYLL → AAAA) of NCoA-1 on rescue of inhibition by microinjected anti-NCoA-1 IgG (α-1) on retinoic-acid- (d) and oestrogen (e)-dependent transcription. In parts c-d, IH1, IH2 etc. represent the equivalent LCDs.

of retinoic acid and IFN-γ gave reciprocal inhibition of retinoic acid- and interferon-dependent reporter gene expression (Fig. 6c). However, the addition of CBP N' P1 peptide relieved inhibition of RAR-dependent transcription by IFN-γ, consistent with the ideas that this inhibition represents, at least in part, competition for CBP co-activator complexes, analogous to that proposed for AP-1 and nuclear receptors³. Our results are consistent with the hypothesis that different 'motifs' are used in assembling CBP-dependent complexes by different classes of transcription factors and can be used to block specific signal-transduction pathways.

Discussion

Our data indicate that p/CIP, a novel factor with which a significant component of the CBP/p300 in the cell is associated, is apparently required for regulated transcription by nuclear receptors and other CBP-dependent factors, including STAT and AP-1. Our findings

show that both the CBP/p/CIP complex and NCoA-1 are required to allow full ligand-activated gene transcription in the cell types that we have examined, whereas NCoA-1/SRC-1 is not required for other CBP-dependent transcription. Because CBP can associate with many additional factors, including Myb (ref. 35), YY1 (ref. 36), SREBP (ref. 37), MyoD and the HLH factors (ref. 9), p/CIP and CBP may be components of a larger complex critical for integration of several signal-transduction pathways. The existence of a co-integrator complex has obvious implications with respect to enhancer-specific functions and expanded ability to respond to diverse regulatory pathways. Whether p/CIP is required for all CBP-dependent transcription factors remains to be established. It has been shown that the N terminus of CBP alone is sufficient to potentiate CREB function in transient co-transfection assays^{38,39}, but the C terminus is also required in transcription assays *in vitro*⁴⁰. Together, our findings suggest that conformational alterations in



specific inhibitory peptides have provided additional evidence that partitioning of CBP^{3,12}, at least in part, accounts for *trans*-repression of nuclear receptor/STAT/AP-1 pathways. The demonstration that specific interaction motifs can selectively block gene activation by specific signal-transduction pathways has potentially intriguing applications to the study of signalling in development, as well as therapeutic implications. □

Transient transfections and reporter assays. Transfection was done in either HeLa or CV-1 cells using the standard calcium phosphate procedure. Typically, 1 μ g RARE- or ERE-driven luciferase reporter was co-transfected with 1 μ g of the indicated vectors. The final DNA concentration was adjusted to 10 μ g per 60-mm dish, incubated for 24 h, and the appropriate ligands administered for 24 h at 10^{-6} M. Alternatively, co-transfections were done using PCMX p/CIP, NCoA-1 or PCR-generated NCoA-1 fragments fused to the

GAL4 DNA-binding domain (residues 1–147). Cells were transfected with 1.0 µg (UAS)₆-luciferase reporter and the indicated concentrations of GAL4 fusion proteins and collected 48 h later.

Generation of affinity-purified NCoA antibodies and peptides. cDNA fragments corresponding to p/CIP(544–851) NCoA-1 (424–789), and NCoA-2(787–1,129) were subcloned into the PM vector containing an in-frame His tag and recombinant His-tagged proteins were generated and purified by nickel chelate chromatography. Purified recombinant proteins were injected into rabbits and antibodies generated and affinity-purified using standard procedures⁴⁵. Peptide sequences, generated (Research Genetics) and confirmed by mass spectroscopy, include: NCoA-1 LCD1 (amino acids 631–647); NCoA-1 LCD2 (687–706); NCoA-1 LCD4 (907–926); CBP N'P1 (1–19); CBP N'P2(8–19).

GST-interaction assays, immunoprecipitations and enzymatic assays. Whole-cell extracts were prepared by lysing cells in NET-N buffer containing 50 mM Tris (pH 7.6), 5 mM EDTA, 0.3 M NaCl, 1 mM DTT, 0.1% NP-40 and protease inhibitors (0.2 mM PMSF, 10 µg ml⁻¹ each of leupeptin, pepstatin and aprotinin), centrifuged at 30K for 1 h at 4 °C; the supernatant was stored at – 80 °C.

GST–RAR(143–462), GST–ER(251–595) and GST–CBP(2,058–2,170) were generated as described³. 25 µl GST–Sepharose beads containing 10 µg GST recombinant proteins were incubated in the presence or absence of the appropriate ligand for 30 min at room temperature, followed by the addition of 1 mg cell extract and incubated for an additional hour at 4 °C. Complexes were then centrifuged, washed three times in NET-N buffer, separated by SDS–PAGE, and western blotted with the appropriate antibodies (1 µg ml⁻¹). For co-immunoprecipitation, 1 mg cell extract was incubated with 2 µg p/CIP or NCoA antibody for 2 h at 4 °C. Immune complexes were then precipitated with protein A–Sepharose (50% w/v). Protein complexes were separated by SDS–PAGE⁴⁶ and western blotted using 1 µg ml⁻¹ of an anti-CBP/p300 monoclonal antibody (UBI). For *in vitro* competition assays, the indicated peptides were incubated with *in vitro* translated NCoA-1 before GST interaction with RAR.

Matagenesis. Mutations in NCoA-1 and CBP were introduced by site-directed mutagenesis using a quick-change mutagenesis kit according to the manufacturer's instructions (Stratagene). Double-stranded oligonucleotides were designed so that the wild-type sequence corresponding to amino acids 695–698 and 756–759 in PCMX NCoA-1 and PJG4-5-4 NCoA-1(635–760) were substituted with alanines. A similar protocol was used to replace amino acids 70–73 in PJG4-5 CBP(1–101).

Single-cell microinjection assay. Insulin-responsive Rat-1 fibroblasts were seeded on acid-washed glass coverslips at subconfluent density and grown in MNE/F12 medium supplemented with 10% fetal bovine serum, gentacin and methotrexate. Before injection, cells were rendered quiescent by incubation in serum-free medium for 24–26 h. Plasmids were injected into the nuclei of cells at 100 µg ml⁻¹. Peptides were injected at 200 µM. Either preimmune IgG of the appropriate species, or antibodies directed against p/CIP, NCoA-1 or NCoA-2, were co-injected and the injected cell unambiguously identified. Microinjections were done using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. About 1 h after injection, cells were stimulated, where indicated, with the appropriate ligand. In rescue experiments, cells were stimulated with ligand 6 h after injection to allow protein expression. After overnight incubation, cells were fixed and stained to detect injected IgG and β-galactosidase expression^{3,47}. Injected cells were identified by staining with tetramethylrhodamine-conjugated donkey anti-rabbit IgG. Data accession numbers of p/CIP and NCoA-2 sequences are AF000581 and AF000582, respectively.

Received 14 January; accepted 6 May 1997.

- Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. & Nakatani, Y. The transcriptional coactivator p300 and CBP are histone acetyltransferases. *Cell* **87**, 953–960 (1996).
- Bannister, A. J. & Kouzarides, T. The CBP coactivator is a histone acetyltransferase. *Nature* **384**, 641–643 (1996).
- Kamei, Y. *et al.* A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 1–12 (1996).
- Yao, T.-P., Ku, G., Zhou, N., Scully, R. & Livingston, D. M. The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. *Proc. Natl Acad. Sci. USA* **93**, 10626–10631 (1996).
- Hanstein, B. *et al.* p300 is a component of an estrogen receptor coactivator complex. *Proc. Natl Acad. Sci. USA* **93**, 11540–11545 (1996).
- Chakravarti, D. *et al.* Role of CBP/p300 in nuclear receptor signalling. *Nature* **383**, 99–103 (1996).
- Kwok, R. P. *et al.* Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370**, 223–226 (1994).
- Arias, J. *et al.* Activation of cAMP and mitogen-responsive genes relies on a common nuclear factor. *Nature* **370**, 226–229 (1994).

- Eckner, R., Yao, T.-P., Oldread, E. & Livingston, D. M. Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation. *Genes Dev.* **10**, 2478–2490 (1996).
- Bhattacharya, S. *et al.* Cooperation of Stat2 and p300/CBP in signalling induced by interferon-α. *Nature* **383**, 344–347 (1996).
- Zhang, J. *et al.* Two contact regions between Stat1 and CBP/p300 in interferon γ signalling. *Proc. Natl Acad. Sci. USA* **93**, 15092–15096 (1996).
- Horvai, A. E. *et al.* Nuclear integration of JAK/STAT and ras signaling by CBP and p300. *Proc. Natl Acad. Sci. USA* **94**, 1074–1079 (1997).
- Chambon, P. The retinoid signaling pathway: molecular and genetic analyses. *Semin. Cell Biol.* **5**, 115–125 (1994).
- Beato, M., Herrlich, P. & Schütz, G. Steroid hormone receptors: many actors in search of a plot. *Cell* **83**, 851–857 (1995).
- Tsai, M. J. & O'Malley, B. W. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* **63**, 451–486 (1994).
- Danielian, P. S., White, R., Lees, J. A. & Parker, M. G. Identification of a conserved region required for hormone-dependent transcriptional activation by steroid hormone receptors. *EMBO J.* **11**, 1025–1033 (1992).
- Durand, B. *et al.* Activation function 2 (AF-2) of retinoic acid receptor and 9-*cis* retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. *EMBO J.* **13**, 5370–5380 (1994).
- Baretino, D., Vivanco Ruiz, M. M. & Stunnenberg, H. G. Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. *EMBO J.* **13**, 3039–3049 (1994).
- Tone, Y., Collingwood, T. N., Adams, M. & Chatterjee, V. K. Functional analysis of a transactivation domain in the thyroid hormone beta receptor. *J. Biol. Chem.* **269**, 31157–31161 (1994).
- Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H. & Moras, D. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. *Nature* **375**, 377–382 (1995).
- Renaud, J.-P. *et al.* Crystal structure of the RAR-γ ligand-binding domain bound to all-*trans* retinoic acid. *Nature* **378**, 681–689 (1995).
- Wagner, R. L. *et al.* A structural role for hormone in the thyroid hormone receptor. *Nature* **378**, 690–696 (1995).
- Halachmi, S. *et al.* Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* **264**, 1455–1458 (1994).
- Cavailles, V. *et al.* Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J.* **14**, 3741–3751 (1995).
- Kurokawa, R. *et al.* Polarity-specific activities of retinoic acid receptors determined by a co-repressor. *Nature* **377**, 451–454 (1995).
- Fondell, J. D., Ge, H. & Roeder, R. G. Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc. Natl Acad. Sci. USA* **93**, 8329–8338 (1996).
- Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A. & Moore, D. D. Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. *Nature* **374**, 91–94 (1995).
- Le Douarin, B. *et al.* The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-ref in the oncogenic protein T18. *EMBO J.* **14**, 2020–2033 (1995).
- Onate, S. A., Tsai, S. Y., Tsai, M.-J. & O'Malley, B. W. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1357 (1995).
- Cavailles, N., Dauvois, S., Danielian, P. S. & Parker, M. G. Interaction of proteins with transcriptionally active estrogen receptors. *Proc. Natl Acad. Sci. USA* **91**, 10009–10013 (1994).
- Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P. & Gronemeyer, H. TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.* **15**, 3667–3675 (1996).
- Hong, H., Kohli, K., Trivedi, A., Johnson, D. L. & Stallcup, M. R. GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc. Natl Acad. Sci. USA* **93**, 4948–4952 (1996).
- Smith, C. L., Onate, S. A., Tsai, M.-J. & O'Malley, B. W. CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. *Proc. Natl Acad. Sci. USA* **93**, 8884–8888 (1996).
- Geourjon, C. & Deleage, G. SOPM: a self optimised prediction method for protein secondary structure prediction. *Protein Eng.* **7**, 157–164 (1994).
- Dai, P. *et al.* CBP as a transcriptional coactivator of c-Myc. *Genes Dev.* **10**, 528–540 (1996).
- Lee, J.-S. *et al.* Relief of YY1 transcriptional repression by adenovirus E1A is mediated by E1A-associated protein p300. *Genes Dev.* **9**, 1188–1198 (1995).
- Oliner, J. D., Andresen, J. M., Hansen, S. K., Zhou, S. & Tjian, R. SREBP transcriptional activity is mediated through an interaction with the CREB-binding protein. *Genes Dev.* **10**, 2903–2911 (1996).
- Bisotto, S., Minogran, S. & Rehms, R. P. Identification and characterization of a novel transcriptional activation domain in the CREB-binding protein. *J. Biol. Chem.* **271**, 17746–17750 (1996).
- Swope, D. L., Mueller, C. L. & Chiriva, J. C. CREB-binding protein activates transcription through multiple domains. *J. Biol. Chem.* **271**, 28138–28145 (1996).
- Nakajima, T., Uchida, C., Anderson, S. F., Parvin, J. D. & Montminy, M. Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors. *Genes Dev.* **11**, 738–747 (1997).
- Yang, X.-Y., Ogryzko, V. V., Nishikawa, J., Howard, B. H. & Nakatani, Y. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**, 319–324 (1996).
- Nakajima, T. *et al.* The signal-dependent coactivator p300 is a nuclear target for pp90 rsk. *Cell* **86**, 465–474 (1996).
- Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**, 791–803 (1993).
- Ausubel, F. M. *et al.* *Current Protocols in Molecular Biology* (Greene, New York, 1994).
- Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988).
- Laemmle, E. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 (1970).
- Rose, D. W., McCabe, G., Feramisco, J. R. & Adler, M. Expression of c-fos and AP-1 activity in senescent human fibroblasts is not sufficient for DNA synthesis. *J. Cell. Biol.* **119**, 1405–1411 (1992).

Acknowledgements. We thank N. Assa-Munt for discussions and help with computer analysis of protein structures; A. Aggarwal, T.-M. Mullen, J. Gemsh and C. Nelson for assistance; M. Parker for the RIP 140 cDNA clone, P. Myer for help in preparing the figures; and B. Stawski for help in preparing the manuscript. This work was supported by an American Diabetes Association career development award (to D.W.R.), by the National Cancer Institute of Canada (J.T.), by a Damon Runyon–Walter Winchell Foundation fellowship (J.L.) and by the Swedish Cancer Society (S.W.). M.G.R. is an investigator with the Howard Hughes Medical Institute. These studies were supported by a US Army Breast Cancer Research Program and grants from the NIH to C.K.G. and M.G.R.

Correspondence should be addressed to D.W.R. and requests for materials to M.G.R.

An asteroidal companion to the Earth

Paul A. Wiegert*, Kimmo A. Innanen* & Seppo Mikkola†

* Department of Physics and Astronomy, York University, 4700 Keele Street, North York, Ontario M3J 1P3, Canada

† Tuorla Observatory, University of Turku, 21500 Piikkiö, Finland

Near-Earth asteroids range in size from a few metres to more than 30 km: in addition to playing an important role in past and present impact rates on the Earth, they might one day be exploited as bases for space exploration or as mineral resources. Many near-Earth asteroids move on orbits crossing that of the Earth, but none has hitherto been identified as a dynamical companion to the Earth. Here we show that the orbit of asteroid 3753 (1986 TO), when viewed in the reference frame centred on the Sun but orbiting with the Earth, has a distinctive shape characteristic of 'horseshoe' orbits. Although horseshoe orbits are a well-known feature of the gravitational three-body problem¹, the only other examples of objects moving on such orbits are the saturnian satellites Janus and Epimetheus²—and their behaviour is much less intricate than that of 3753. Moreover, the fact that 3753 exhibits such a dynamical relationship with the Earth shows that, although it is not a satellite of our planet *per se*, it is, apart from the Moon, the only known natural companion of the Earth.

Figure 1 shows a view of the inner Solar System projected onto the ecliptic plane in a frame which co-rotates with the Earth; in this frame, the Earth appears stationary. The path of asteroid 3753 over a little more than one year is indicated by the line with arrowheads. The path has roughly the shape of a kidney bean, owing simply to the eccentricity of the asteroid's orbit. As the asteroid's orbital period is currently slightly shorter than that of the Earth, its orbit does not close on itself but rather advances slightly each year: the asteroid thus spirals forward along the orbit of the Earth.

This behaviour is not unusual in itself. What distinguishes 3753 from other near-Earth asteroids (NEAs) is its behaviour as it approaches the Earth: our planet's gravitational pull acts to increase the asteroid's period from slightly below to slightly above one year. As a result, the asteroid begins to fall behind, and hence to move away from the Earth. A possible collision with our planet is avoided: the closest approach during this leg is indicated by the heavy line A in Fig. 1.

Having reversed its direction, the asteroid eventually approaches the Earth from the other side. In this case, however, the Earth acts to decrease the asteroid's period to its previous value slightly below one year, and 3753 begins moving away from the Earth again. At closest approach on this leg, the edge b of the 'kidney bean' trajectory coincides with the heavy line labelled B in Fig. 1. The cycle of reversals then goes on to repeat itself, the Earth effectively repelling the asteroid at each close approach. The previous two reversals occurred in approximately AD 1515 and 1900; the next two will occur in AD 2285 and 2680. The variation in semimajor axis of 3753 over the next 2,000 years is shown in Fig. 2, the reversals corresponding to transitions across the semimajor axis of the Earth (1 astronomical unit, AU, the average Earth–Sun distance).

It should be noted that reversals occur when edge a (not b; see Fig. 1) approaches the Earth. Owing to the inclination and orientation of the asteroid's orbit, edge b never approaches the Earth closely, despite its appearance when projected onto the ecliptic plane. Figure 3 presents a view of the inner Solar System from an ecliptic edge-on perspective, and the asteroid's high inclination (20°) is evident. The orientation of the asteroid's orbit allows edge b to overlap the position of the Earth in Fig. 1 with no danger of

collision. The complete orbit of 3753 is thus an 'overlapping horseshoe' (the outer envelope of which is indicated by the heavy line in Fig. 1), a kind which has never been observed before, even in theoretical studies. The asteroid's significant inclination and eccentricity ($e \approx 0.5$) have evidently caused it to escape more intensive scrutiny since its discovery, as they serve effectively to obscure the nature of its trajectory.

The asteroid's orbit is chaotic, on a timescale of 150 years, but it remains a near-Earth object in our simulations for timescales of a million years. However, not all of this time is spent in a horseshoe orbit: the asteroid can switch between its current orbit and a non-horseshoe orbit with semimajor axis around 1.1 AU on timescales of a few hundred thousand years.

Asteroid 3753 passes from inside to outside the Earth's orbit, but its minimum yearly approach distance is usually quite large. During the closest approaches, which happen only every 385 years, the asteroid passes within 0.1 AU (roughly 40 times the Earth–Moon distance) of our planet, the last such approach having occurred about 100 years ago. Over the next year, the closest approach will be only to within 0.31 AU. The relative proximity of 3753 to the Earth during recent times presumably aided in its discovery by Waldron in October 1986^{3,4}. Asteroid 3753 has an absolute visual magnitude of 15.1 (ref. 5), brighter than typical of NEAs, and from which one can estimate a diameter of 5 km (refs 6, 7).

Asteroid 3753 crosses the orbits of Venus and Mars as well as that of the Earth. Though its orbit does not currently intersect that of any planet, the asteroid's argument of perihelion precesses at a rate of roughly $\dot{\omega} \approx +0.6^\circ$ per century. As a result, its orbit will intersect that of the Earth in 2,750 years, and (if it survives this crossing) that of Venus in about 8,000 years. Similarly, the asteroid's orbit intersected Mars's roughly 2,500 years ago. These results suggest that the asteroid's current horseshoe orbit may not be stable for arbitrarily long times, unless there is some dynamical 'safety mechanism' which preserves it against close planetary encounters. At this point, the existence of such a safety mechanism seems unlikely, and yet the very low a priori probability of an object being injected

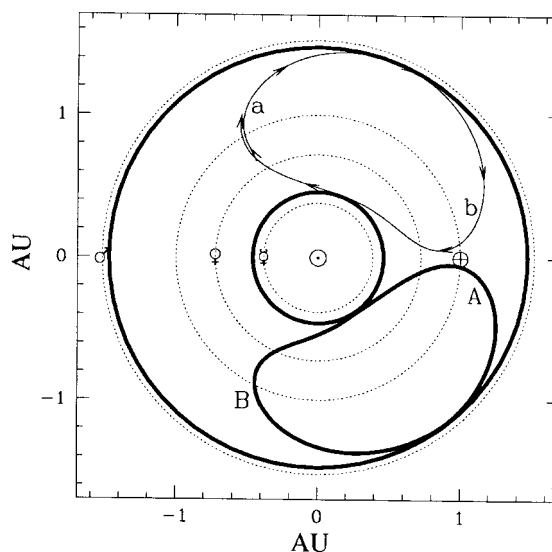


Figure 1 A view of the inner Solar System projected onto the ecliptic plane in a frame which co-rotates with the Earth; in this frame, the Earth appears stationary and is located at the symbol ⊕. The average distances of the planets Mercury, Venus, Earth and Mars from the Sun are indicated by dotted circles. The path of asteroid 3753 over slightly more than a year, beginning approximately in AD 2000, is shown by the line with arrowheads, with the outer envelope of the horseshoe indicated by the heavy lines. Reversals occur when a coincides with A, and when b coincides with B (see text).

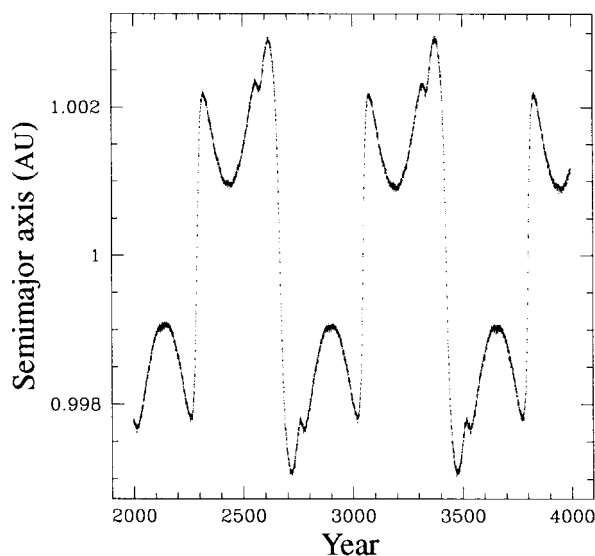


Figure 2 The heliocentric semimajor axis a of asteroid 3753 (1986 TO) over the next 2,000 years. As the asteroid's orbital period is proportional to $a^{3/2}$, transitions from $a > 1$ AU to $a < 1$ AU or vice versa correspond to reversals of the asteroid's direction within the horseshoe.

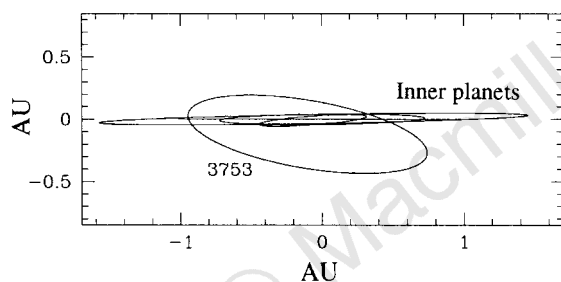


Figure 3 The orbits of the inner planets Mercury, Venus, Earth and Mars, along with that of asteroid 3753 when seen from the direction of the vernal equinox. The orbits of the inner planets are hard to distinguish, but the relatively high inclination of 3753 is apparent. The Sun is at the origin.

into such an unusual orbit makes a recent origin seem equally unlikely. As for the asteroid's future, though prediction is problematic owing to 3753's short chaotic timescale, a collision with the Earth seems very improbable. A strong gravitational interaction with Venus in 8,000 years seems quite likely, though the possibility of a collision with that planet at that time remains remote. On balance therefore, the origin of this object remains an enigma. More detailed investigation of this object is clearly required; even the direct exploration of 3753 by spacecraft is presumably well within the reach of current technology. □

Methods

The numerical simulations of asteroid 3753 presented here were performed with the Wisdom–Holman⁸ integrator, in a model Solar System which included all the planets except Pluto. It should be noted however that the Earth–Moon barycentre was used for the Earth, an approximation which is valid because the Earth–asteroid distance is always much larger than the Earth–Moon distance. The heliocentric orbital elements of 3753 used were⁵: semimajor axis $a = 0.99778030$ AU, eccentricity $e = 0.51478431$, inclination $i = 19.812285^\circ$, longitude of the ascending node $\Omega = 126.373212^\circ$, argument of perihelion $\omega = 43.640637^\circ$, and mean anomaly $M = 40.048932^\circ$; these elements were calculated for epoch JD 2450500.5 and the equinox of J2000.0.

Received 10 February; accepted 1 April 1997.

1. Szebehely, V. *Theory of Orbits* (Academic, New York, 1967).
2. Dermott, S. F. & Murray, C. D. The dynamics of tadpole and horseshoe orbits. II The coorbital satellites of Saturn. *Icarus* **48**, 12–22 (1981).
3. *Minor Planet Circular 11312* (Minor Planet Center, Smithsonian Astrophys. Observ., Cambridge, MA, 1986).
4. *IAU Circ. No. 4262 and 4266* (1986).
5. Bowell, E. *The Asteroid Orbital Elements Database* available at <ftp://ftp.lowell.edu/pub/elgb/astorb.html> (1997).
6. Bowell, E. *et al.* in *Asteroids II* (eds Binzel, R. P. *et al.*) 524–556 (Univ. Arizona Press, Tucson, 1989).
7. Rabinowitz, D. L. The size distribution of earth-approaching asteroids. *Astrophys. J.* **407**, 412–427 (1993).
8. Wisdom, J. & Holman, M. Symplectic maps for the n -body problem. *Astron. J.* **102**, 2022–2029 (1991).

Acknowledgements. We thank M. Holman for the use of his integration codes. This work was supported in part by the Natural Sciences and Engineering Research Council of Canada.

Correspondence should be addressed to P.A.W. (e-mail: wiegert@yorku.ca).

A silicon/iron-disilicide light-emitting diode operating at a wavelength of $1.5 \mu\text{m}$

D. Leong, M. Harry, K. J. Reeson & K. P. Homewood

Department of Electronic and Electrical Engineering, University of Surrey, Guildford, Surrey GU2 5XH, UK

Although silicon has long been the material of choice for most microelectronic applications, it is a poor emitter of light (a consequence of having an 'indirect' bandgap), so hampering the development of integrated silicon optoelectronic devices. This problem has motivated numerous attempts to develop silicon-based structures with good light-emission characteristics¹, particularly at wavelengths ($\sim 1.5 \mu\text{m}$) relevant to optical fibre communication. For example, silicon–germanium superlattice structures² can result in a material with a pseudo-direct bandgap that emits at $\sim 1.5 \mu\text{m}$, and doping silicon with erbium³ introduces an internal optical transition having a similar emission wavelength, although neither approach has led to practical devices. In this context, β -iron disilicide has attracted recent interest^{4–12} as an optically active, direct-bandgap material that might be compatible with existing silicon processing technology. Here we report the realization of a light-emitting device operating at $1.5 \mu\text{m}$ that incorporates β -FeSi₂ into a conventional silicon bipolar junction. We argue that this result demonstrates the potential of β -FeSi₂ as an important candidate for a silicon-based optoelectronic technology.

There has been interest for some time in the semiconducting β -phase of iron disilicide as a possible route to optically active structures compatible with silicon technology. However, experimental investigations into this material have been limited and even its basic properties, such as the value of the bandgap energy and the type (that is, the directness), have been controversial^{4–12}. Photoluminescence has also been observed but its origin has been disputed and has been attributed by some^{13,14} to defects in the surrounding silicon. Recent work^{15,16} has shown that β -iron disilicide, β -FeSi₂ (produced by ion implantation into device-quality (100) silicon followed by suitable annealing) is direct-gap. Photoluminescence at a wavelength of $1.5 \mu\text{m}$ has been observed in this material and has been shown conclusively to be band-edge-related luminescence from the β -iron disilicide¹⁷.

Efficient semiconducting electroluminescent sources such as high-intensity light-emitting diodes (LEDs) and particularly injection lasers are made from direct-gap semiconductors, as these provide an efficient and fast radiative route. It is also necessary to incorporate heterojunctions to provide good carrier and optical

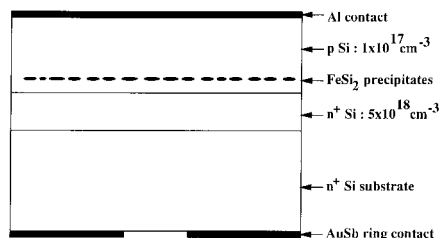


Figure 1 Schematic of the LED structure used. Further details of the structure are provided in the text.

confinement and to enable efficient light extraction. β -FeSi₂ now seems to be able to offer all these properties and so this material would seem a promising route to practical optical sources in silicon. The use of ion beam synthesis to fabricate such a device is particularly attractive, as the use of many ion implantation stages is ubiquitous in the production of integrated silicon circuits, and so this technique is well suited to current silicon process technology; we note that customized implantation equipment may have to be used as was done in the case of silicon implanted with oxygen (SIMOX) technology.

We have achieved, and describe here, the successful fabrication of a demonstrator silicon/iron-disilicide light-emitting diode. The approach used is to incorporate direct-gap iron disilicide into a conventional silicon p-n junction diode, in the recombination region adjacent to one side of the depletion region, to provide a route for direct radiative recombination. Carrier injection is achieved conventionally under forward bias. A schematic of this device is shown in Fig. 1. Initially, to avoid unnecessary complications, the implantation of the iron to form the buried silicide region was made into a previously grown abrupt silicon p-n junction. The silicon p-n junction was grown by molecular beam epitaxy and consisted of a boron-doped p-type region, 1.0 μm thick, grown on a 0.4- μm -thick antimony doped n-type region; the doping densities in the two regions were $1 \times 10^{17} \text{ cm}^{-3}$ and $5 \times 10^{18} \text{ cm}^{-3}$, respectively. These epilayers were grown on an n-type (100) silicon substrate with a resistivity of 0.008–0.02 $\Omega \text{ cm}$. Ultimately it is anticipated that the whole device including the p-n junction could be fabricated entirely by ion implantation. The implant dose used, $2 \times 10^{16} \text{ cm}^{-2}$, produces isolated precipitates of single-crystal β -FeSi₂ on annealing. A continuous buried iron silicide layer can be formed if higher implant doses are used¹⁸, but we believe the use of precipitates is preferable as the implantation time and consequently the cost of device production would be reduced dramatically. Moreover, this type of structure could be realized with commercial high-current ion implanters. The precipitates are single crystal and have typical dimensions of several hundred $\text{\AA}$, so no quantum confinement effects are expected¹⁷. The implant energy, 950 keV, was chosen to place the peak of the precipitate distribution above the depletion region of the silicon p-n junction.

After implantation at 350 °C, the device was annealed at 900 °C for 18 hours in an optical lamp furnace in a nitrogen atmosphere, a procedure that was previously found to give good photoluminescence⁶ but also moderate enough for diffusion of the junction dopants to be minimal; we estimate diffusion lengths for the Sb and B of $\sim 50 \text{ nm}$. Ohmic contacts, 1 mm in diameter, consisting of Al and AuSb eutectic were deposited by vacuum evaporation and then alloyed onto the p-type region and the n-type substrate, respectively. A window was left in the back contact to allow passage of the electroluminescence (EL) through the transparent silicon substrate. The diodes were 'isolated' by mesa etching down to the substrate material¹⁹. Current–voltage (*I*–*V*) measurements were then made to check for diode integrity. A plot of the *I*–*V* characteristic of a fully processed and working device is shown in

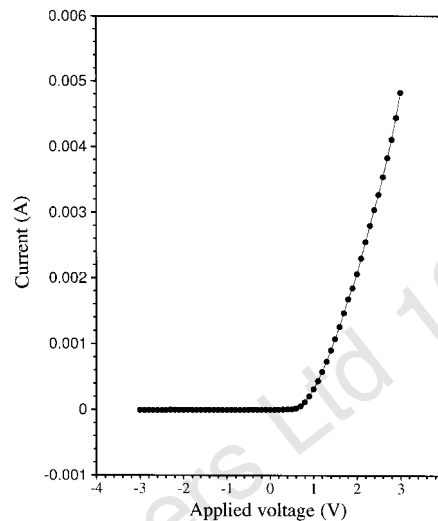


Figure 2 The current–voltage plot for the working device measured at room temperature; the reverse leakage current is 3 μA .

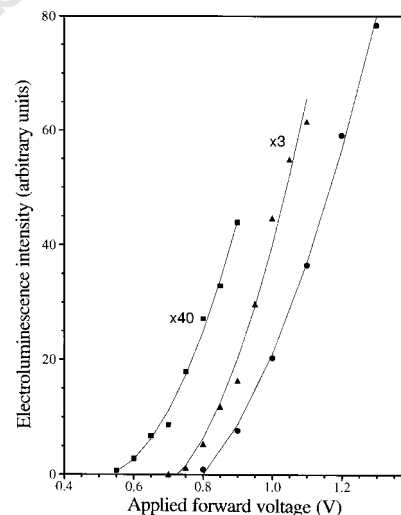


Figure 3 Plots of the integrated electroluminescence intensity as a function of applied forward voltage at various temperatures: filled circles, 80 K; filled triangles, 180 K; and filled squares, 300 K. The solid lines are provided as guides to the eye.

Fig. 2. The good characteristics obtained demonstrate that the diode integrity has been retained throughout the device processing steps. Individual devices were separated and mounted in a variable-temperature, dynamic continuous-flow liquid nitrogen cryostat placed in front of a conventional half-metre spectrometer. A liquid nitrogen cooled germanium p-i-n diode was used for detection of the EL.

Initial EL measurements were made at 80 K. The onset of EL was seen when the diode was forward biased to around 0.8 V. The intensity of the EL emission as a function of forward bias, at various measurement temperatures, is shown in Fig. 3; the form of the curves and the values of the turn-on voltages are entirely consistent with conventional injection across the p-n junction under forward bias being the excitation mechanism. The EL spectrum taken at a forward current of 15 mA is shown in Fig. 4. The EL measured at 80 K peaks at a wavelength of 1.54 μm and has a full width at half height of 50 meV. In Fig. 5 we show the temperature dependence of the EL intensity between 80 K and room temperature. The EL

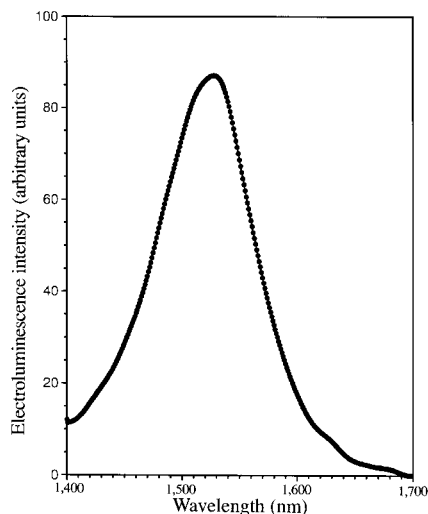


Figure 4 Spectrum of electroluminescence intensity against wavelength, measured at 80 K. The forward current through the device was 15 mA.

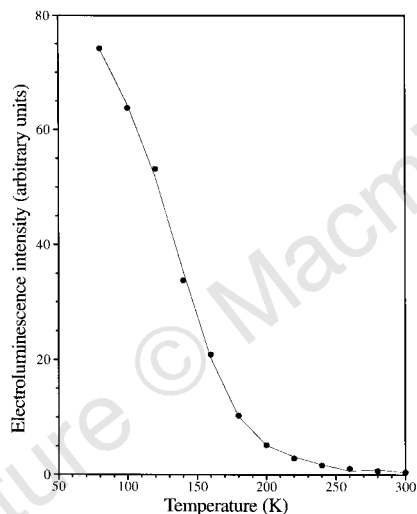


Figure 5 A plot of the integrated intensity as a function of measurement temperature. All measurements were made at a forward current of 15 mA. The solid line is provided as a guide to the eye.

reduces with increasing temperature but could still be seen clearly at room temperature. The peak energy of the EL shifts slightly towards lower energies with increasing temperature, as expected for a band-edge-related emission. The shift in peak energy with temperature, ~ 50 meV between 80 and 300 K, follows precisely that observed previously for the bandgap shift of the iron disilicide determined from an optical absorption study¹⁵.

The device was subjected to frequent temperature cycling between room temperature and 80 K; it has operated in continuous wave mode for several hundred hours. Significantly, no changes or deterioration in the EL quality, intensity or operating conditions have so far been observed.

We have demonstrated the fabrication of a working and robust LED in silicon, based on β -FeSi₂; this LED is not just an important device in itself but is also significant as an essential first step for the subsequent development of a 1.5- μ m injection laser in silicon. The quantum efficiency of our device is low; we estimate $\sim 0.1\%$,

depending on the particular operating conditions. Nevertheless, there appear to be, so far, no fundamental reasons why optimization of the device design and material quality could not lead to greatly improved efficiencies. □

Received 29 October 1996; accepted 29 April 1997.

1. Miller, D. A. Silicon sees the light. *Nature* **378**, 238 (1995).
2. Forster, M. *et al.* Photoluminescence and photocurrent studies of Si/SiGe p-i-n heterostructures. *J. Appl. Phys.* **80**, 3017–3023 (1996).
3. Zheng, B. *et al.* Room temperature sharp line luminescence at 1.5 microns from an erbium doped light emitting diode. *Appl. Phys. Lett.* **64**, 2842–2844 (1994).
4. Christensen, N. E. Electronic structure of beta-FeSi₂. *Phys. Rev. B* **42**, 7148–7153 (1990).
5. Eppenga, R. Ab initio band structure calculation of the semiconductor beta-FeSi₂. *J. Appl. Phys.* **68**, 3027–3029 (1990).
6. Finney, M. S. *et al.* Effects of annealing and cobalt implantation on the optical properties of beta-FeSi₂. *Mater. Res. Soc. Symp. Proc.* **316**, 433–438 (1994).
7. Radermacher, K., Skeide, R., Carius, J., Klomfass, J. & Mantl, S. Electrical and optical properties of FeSi₂ layers. *Mater. Res. Soc. Symp. Proc.* **320**, 115–120 (1994).
8. Giannini, C., Lagomarsino, S., Scarinci, F. & Castrucci, P. Nature of the bandgap of polycrystalline beta-FeSi₂ films. *Phys. Rev. B* **45**, 8822–8824 (1992).
9. Rosen, B. N. E. *et al.* Characterisation of beta-FeSi₂/Si heterostructures grown by gas source MBE. *Mater. Res. Soc. Symp. Proc.* **320**, 139–144 (1994).
10. Bost, M. C. & Mahan, J. E. A clarification of the index of refraction of beta iron disilicide. *J. Appl. Phys.* **64**, 2034–2037 (1988).
11. Bost, M. C. & Mahan, J. E. Optical properties of semiconducting FeSi₂ films. *J. Appl. Phys.* **58**, 2696–2703 (1985).
12. Dimitriadis, C. A. *et al.* Electronic properties of semiconducting FeSi₂ films. *J. Appl. Phys.* **68**, 1726–1734 (1990).
13. Sauer, R. *et al.* Dislocation related photoluminescence in silicon. *Appl. Phys. A* **36**, 1–13 (1985).
14. Radermacher, K., Carius, R. & Mantl, S. Optical and electrical properties of buried semiconducting beta-iron disilicide S. *Nucl. Instrum. Meth. B* **84**, 163–167 (1994).
15. Yang, Z., Homewood, K. P., Finney, M. S., Harry, M. & Reeson, K. J. Optical absorption study of ion beam synthesized polycrystalline semiconducting FeSi₂. *J. Appl. Phys.* **78**, 1958–1963 (1995).
16. Katsumata, H. *et al.* Optical absorption and photoluminescence studies of beta-FeSi₂ prepared by heavy implantation of Fe⁺ ions into silicon. *J. Appl. Phys.* **80**, 5995–5962 (1996).
17. Leong, D., Harry, M., Reeson, K. J. & Homewood, K. P. On the origin of the 1.5 micron luminescence in ion beam synthesized beta-FeSi₂. *Appl. Phys. Lett.* **68**, 1649–1650 (1996).
18. Hunt, T. D. *et al.* Optical properties and phase transformations in alpha and beta iron disilicide layers. *Nucl. Instrum. Meth. B* **84**, 168–171 (1994).
19. Reeson, K. J. *et al.* Electrical and optical properties of ion beam synthesised (IBS) FeSi₂. *J. Nucl. Instrum. Meth. B* **106**, 364–371 (1995).

Acknowledgements. We thank E. Parker and P. J. Phillips of the University of Warwick for growing the silicon p-n junction layers. This work was supported by the UK Engineering and Physical Sciences Research Council.

Correspondence and requests for materials should be addressed to K.P.H. or K.J.R. (e-mail: k.homewood@surrey.ac.uk; k.reeson@ee.surrey.ac.uk).

Single-molecule optical switching of terrylene in *p*-terphenyl

F. Kulzer, S. Kummer, R. Matzke, C. Bräuchle & Th. Basché*

Institut für Physikalische Chemie, Universität München, Sophienstrasse 11, 80333 München, Germany

The controlled manipulation and switching of single atoms and molecules raise the prospect of ultra-high-density data storage. Switching by motion of a single atom has been reported¹, and techniques of single-molecule optical detection and spectroscopy² in the condensed phase have been refined to a degree that allows the modification of the absorption properties of a single chromophore³. Light-induced jumps in single-molecule excitation frequencies have been reported^{3–5}, but in none of these cases could the process be controlled: the jumps varied from molecule to molecule, they were interrupted by spontaneous jumps, and the new excitation frequencies could not be identified unambiguously. Here we report light-induced reversible frequency jumps of single molecules of the aromatic hydrocarbon terrylene embedded in a particular site of a *p*-terphenyl host crystal⁶ at temperatures of around 2 K. The changes in absorption frequency

* Present address: Institut für Physikalische Chemie, Johannes-Gutenberg-Universität, 55099 Mainz, Germany.

for different terrylene molecules were identical (within 0.5%) for all samples studied. Thus we were able to switch single-molecule absorption lines in a controlled way between well-defined frequency positions.

Thin platelets with very low dopant concentration were prepared by co-sublimation of *p*-terphenyl and terrylene. This system shows four prominent purely electronic origin states, denoted X_1 – X_4 and attributed to four different crystalline insertion sites of hitherto unknown structure. The spectral dynamics depends strongly on the site^{6,7}. Although single molecules in X_2 are very stable with respect to changes in absorption frequency^{6,8} we find reversible, light-induced frequency jumps in site X_1 (absorption wavelength $\lambda_{\text{abs}} = 580.4$ nm).

Figure 1 illustrates this behaviour for two molecules. The fluorescence excitation spectra (Fig. 1a) show both molecules at their initial spectral positions in X_1 . When an excitation intensity of 0.25 W cm^{-2} is applied at the absorption maximum of the molecule marked with an asterisk, this molecule will stay in resonance for typically 10–60 s and then it will undergo a spectral jump (the

single-molecule analogy of spectral hole-burning⁹) of 843 GHz to a new spectral position at a shorter wavelength which we call photo-site XY (Fig. 1b). This is an enormous jump width for single-molecule spectroscopy—more than 10,000 times greater than the homogeneous line width of terrylene in *p*-terphenyl. On resonant excitation in XY the molecule returns to its original spectral position which can be shown with high accuracy as the second molecule serves as a frequency marker during the jump cycle (Fig. 1c). This second molecule exhibits the same spectral-jump behaviour; when both molecules are coerced to XY they show the same spectral pattern as in X_1 (Fig. 1d, e), indicating identical jump widths for both molecules.

So far we have investigated ~50 molecules in seven different crystals taken from four sublimation runs. In 'low-concentration' samples (meaning less than five molecules in the $5\text{-}\mu\text{m}$ excitation spot) we have studied X_1 molecules with resonance frequencies from 580.368 to 580.405 nm and always found the same reversible jumps over 843 ± 2 GHz, with a variation of at most 500 MHz for molecules in the same crystal. The light-driven nature of the process

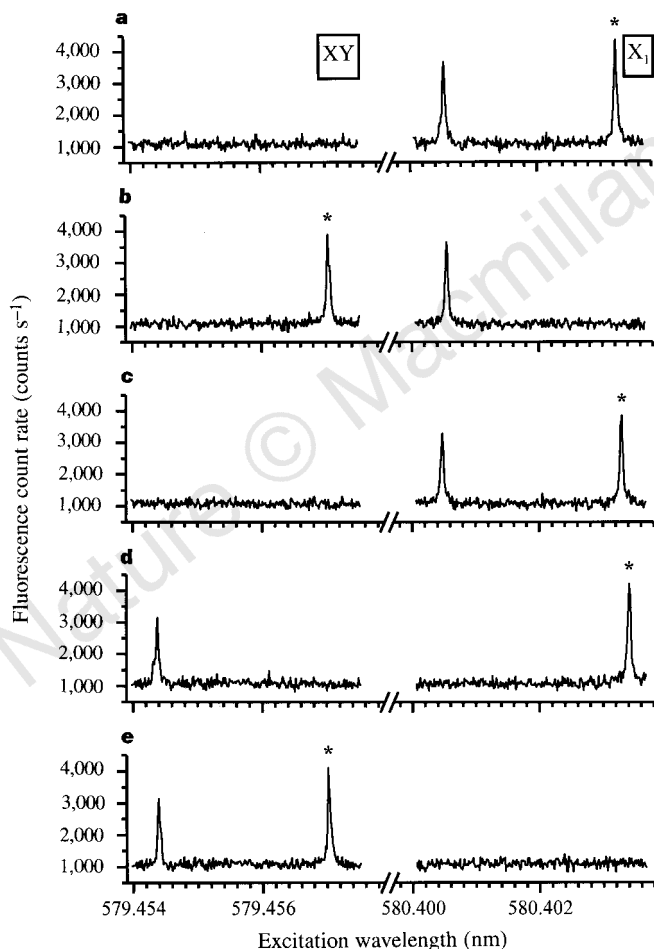


Figure 1 Light-induced, reversible frequency jumps of the fluorescence excitation lines of two single terrylene molecules in a *p*-terphenyl crystal at $T = 1.4$ K. The two different spectral positions (sites) which the molecules occupy are denoted X_1 and XY. The content of the five traces is described in detail in the text. The laser intensity to scan the excitation lines was 0.25 W cm^{-2} . Frequency jumps were induced by resonant pumping for typically 10–60 s with the same intensity. Note the break in the wavelength axis.

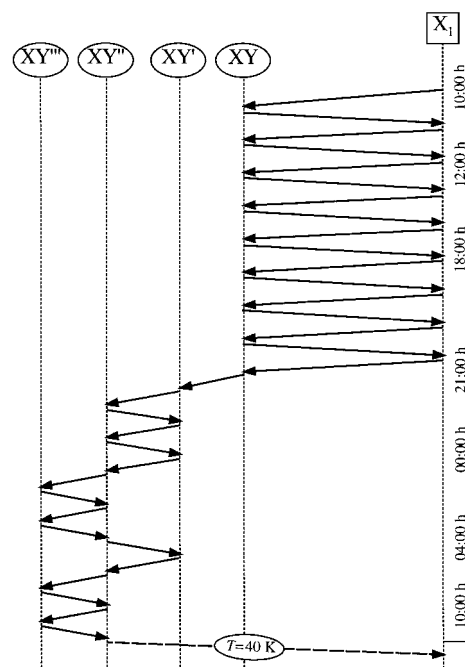


Figure 2 24-hour spectral trace of the terrylene molecule marked with an asterisk in Fig. 1 ($T = 1.4$ K). We found that after eight light-induced X_1 –XY jump cycles the molecule 'burned' from XY to XY'. From then on we found similar light-driven jump cycles between XY' and XY'' as well as between XY'' and XY'''. No light-induced jumps back to XY or X_1 were observed, but the molecule was finally reset to its original X_1 spectral position when the temperature was raised to 40 K. The fairly long time intervals between individual jumps are determined by the time taken to tune the actively stabilized dye-laser to the various frequency positions. The trace presented here is part of a 'spectral diary' where the light-induced frequency jumps of the molecule were followed for 22 days.

was proved unambiguously by intensity-dependence measurements which showed a decrease in burning-time when the light intensity was raised.

Apart from this predominant bistable jump cycle, there are additional spectral positions which can be reproduced for different molecules almost as well as the XY photosite. We conducted a detailed investigation over a long period of time of the two terrylene molecules whose fluorescence excitation spectra are shown in Fig. 1. Because these molecules were well isolated—both spatially and spectrally—from all other terrylene molecules in the sample, we could unambiguously identify them from day to day; we studied them for 22 days. By doing this we could record the behaviour of the molecule whose absorption peak is marked with an asterisk in Fig. 1, and write a kind of 'spectral diary'. For this molecule we found three additional spectral positions which we denoted XY' ($\lambda_{\text{abs}} = 579.153 \text{ nm}$), XY'' ($\lambda_{\text{abs}} = 578.828 \text{ nm}$) and XY''' ($\lambda_{\text{abs}} = 578.556 \text{ nm}$). Figure 2 contains detailed information about the light-induced frequency jumps of this molecule during 24 hours (part of the 22-day observation period). On that day, the molecule underwent eight light-driven X_1 -XY jump cycles, then it vanished from XY to appear at XY'. From then on, we found reversible light-induced jump cycles between XY'-XY'' and XY''-XY'''. During this time there were no direct transitions XY'''-XY' and no light-driven jumps back to XY or X_1 . The molecule was finally 'reset' to its X_1 spectral position by raising the temperature above 40 K.

These additional spectral positions could be found for all X_1 molecules we have investigated so far: typically, the molecules undergo five to ten of the X_1 -XY jump cycles and then they burn

away to XY' after resonant excitation in XY. Furthermore, it appears that when driving a molecule from X_1 to XY''' this process always occurs successively via XY, XY' and XY'' without skipping any of the positions. All positions are highly reproducible over a long time, so that a given X_1 molecule can be observed at its various spectral positions for several weeks at least. The molecules can always be 'reset' to their original spectral positions in X_1 by raising the temperature of the sample to 40 K for a few seconds and cooling down again. Thus X_1 seems to be the most stable spectral position and we never find any molecules in the other photosites (in low-concentration crystals) without prior burning in X_1 .

To get an insight into the mechanism of the light-induced frequency jumps, we measured the vibrationally resolved emission spectra at X_1 and at three of the photosites (using an apparatus described in detail in ref. 10). As under prolonged irradiation a single molecule will quickly jump out of resonance, making it impossible to record its emission spectrum with sufficient signal-to-noise ratio (SNR), we simultaneously had to acquire the signal from ~ 10 different molecules. The whole set was sent repeatedly to the various spectral positions by light irradiation. Figure 3a displays the low-frequency part of the emission spectra which is characterized by two strong vibronic lines at 244 and 253 cm^{-1} . According to quantum-mechanical calculations for planar terrylene¹⁰ and as was found for terrylene in polyethylene^{10,11}, there should be only one low-frequency vibration (long-axis stretch of whole molecule¹⁰) in this range. The insertion of terrylene into the *p*-terphenyl crystal is thought to distort the molecular geometry inducing intensity into a non-totally symmetric mode¹². It is in this low-frequency range of the emission spectra where pronounced differences were observed

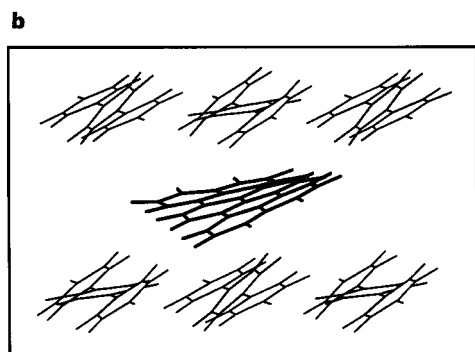
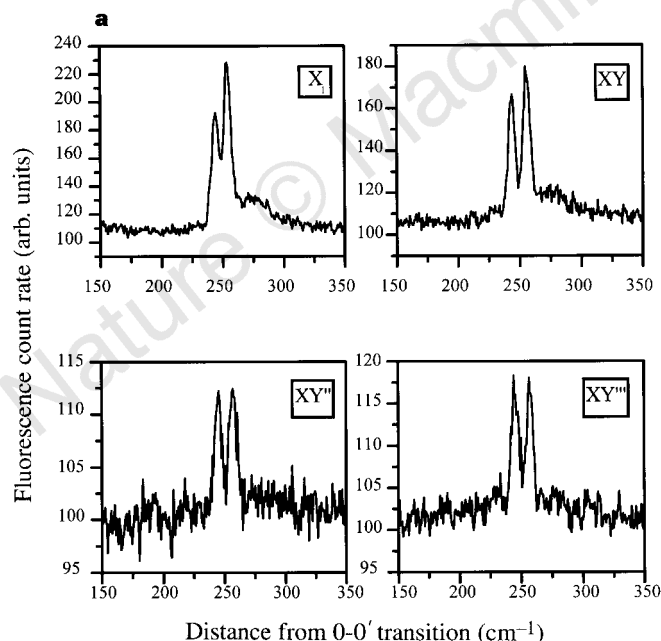


Figure 3 a, Low-frequency part of the fluorescence emission spectra of terrylene in *p*-terphenyl at $T = 2.4 \text{ K}$ excited at the 0-0' transition frequencies of the sites X_1 , XY, XY'' and XY'''. The following procedure was employed to acquire the spectra. A set of approximately ten different molecules found in the spectral region of X_1 was scanned rapidly by the laser and its fluorescence emission after passing a 0.45-m spectrograph detected with a liquid-N₂ cooled CCD camera. Typically, most of the molecules were 'burned' to XY by the scanning laser after several minutes. Then the emission spectrum at XY was recorded using the same procedure. The other two photosites were also populated by light irradiation, and their emission spectra acquired. To achieve sufficient signal-to-noise ratio, the molecules had to be sent repeatedly to the various sites; the overall time for recording a spectrum at a specific site was $\sim 50 \text{ min}$. Because the photostability varies from site to site the corresponding spectra are noisier in the less-stable sites (XY'', XY''') where the molecules 'burn away' quickly. **b**, Schematic representation of the guest site configuration of terrylene in *p*-terphenyl according to a molecular packing calculation¹¹. Optical excitation of terrylene is thought to induce conformational changes from the helicoidally twisted ground-state structure shown here to the planar structure favoured by the electronically excited state¹¹. Our model proposes that under prolonged excitation these repeated conformational changes will eventually induce a central ring flip of an adjacent *p*-terphenyl molecule. This in turn alters the dipolar host-guest coupling and shifts the absorption frequency of the chromophore.

between the different sites of the jump cycle. In Fig. 3a it is seen that when the molecules are shifted spectrally from X_1 to XY the intensity ratio of the two low-frequency lines changes appreciably. This effect was verified for the X_1 – XY jump cycle with three other sets of molecules. Within our resolution limits ($\pm 3\text{ cm}^{-1}$), no alteration of the vibrational frequencies was observed.

The differences in the intensity ratio of the two low-frequency lines at the various spectral positions of the jump cycle may be caused by a slight additional distortion of the terrylene skeleton or by a variation of the local environment triggered by the photo-excitation, or both. Although a conformational change of terrylene may well be involved in the process, we believe that the dominant contribution results from the p -terphenyl environment, which is known to possess a specific reorientational degree of freedom¹³. In a recent model¹⁴ the spontaneous frequency fluctuations (spectral diffusion) of single pentacene optical excitation lines in a p -terphenyl crystal¹⁵ were attributed to a reorientation of the central phenyl ring of p -terphenyl molecules in the nanoenvironment of the chromophores. This phenyl-ring motion is thought to occur in an asymmetric double-well potential. Along similar lines we can tentatively devise the following model for the mechanism of light-induced frequency jumps of single terrylene molecules (Fig. 3b). By promoting the molecule to the electronically excited state a change of the geometry of terrylene is induced. This assumption is supported by the drastically diminished relative intensity of the lower-frequency vibronic line (244 cm^{-1}) in the excitation spectrum (not shown here^{6,12}). Triggered by the geometry change of the chromophore, a next-neighbour p -terphenyl molecule might alter the orientation of its central phenyl ring, leading to a new local environment of the chromophore which (by modification of the dipolar host–guest coupling) changes its absorption frequency. This sequence of events could account for the frequency jump of a molecule from X_1 to XY . By excitation at the new spectral position (XY) the situation may be reversed. The additional spectral positions accessible from XY may be reached by a reorientation of the central phenyl ring of other p -terphenyl molecules in the next solvent shell of the chromophore, indicated by the smaller jump widths. If this tentative model proves to be correct we have the intriguing result that excitation of a single molecule can be used to reversibly change the nanoscopic structure of a molecular solid in a well-defined way.

The light-induced frequency jumps described here are unique in the sense that we find the same behaviour for every single molecule even in different (low-concentration) crystals. Therefore, we can prepare as many ‘copies’ of the same jump cycle as we want and, as we can conduct long-term investigations of one absorber, there seems to be a fair probability that we can eventually gain understanding of the mechanism of photophysical hole-burning on a truly molecular level. Although many obstacles still exist, this observation of reversible, reproducible frequency jumps might pave the way towards the design of host–guest systems suitable for optical switching and storage functions at the single-molecule level. □

Received 24 January; accepted 22 April 1997.

1. Eigler, D. M., Lutz, C. P. & Rudge, W. E. An atomic switch realized with the scanning tunnelling microscope. *Nature* **352**, 600–603 (1991).
2. Basché, Th., Moerner, W. E., Orrit, M. & Wild, U. P. (eds) *Single-Molecule Optical Detection, Imaging and Spectroscopy* (VCH, Weinheim, 1997).
3. Basché, Th. & Moerner, W. E. Optical modification of a single impurity molecule in a solid. *Nature* **355**, 335–337 (1992).
4. Fleury, L., Zumbusch, A., Orrit, M., Brown, R. & Bernard, J. Spectral diffusion and individual two-level systems probed by fluorescence of single terrylene molecules in a polyethylene matrix. *J. Lumin.* **56**, 15–28 (1993).
5. Moerner, W. E. *et al.* Optical probing of single molecules of terrylene in a Shpol'skii matrix: a two-state single molecule switch. *J. Phys. Chem.* **98**, 7382–7389 (1994).
6. Kummer, S., Basché, Th. & Bräuchle, C. Terryene in p -terphenyl: a novel single crystalline system for single molecule spectroscopy at low temperatures. *Chem. Phys. Lett.* **229**, 309–316 (1994); *Chem. Phys. Lett.* **232**, 414 (1995).
7. Kador, L., Müller, A. & Richter, W. Single molecule spectroscopy and persistent hole-burning of terryene in p -terphenyl: external field effects. *Mol. Cryst. Liq. Cryst.* **291**, 23–29 (1996).
8. Basché, Th., Kummer, S. & Bräuchle, C. Direct spectroscopic observation of quantum jumps of a single molecule. *Nature* **373**, 132–134 (1995).

9. Moerner, W. E. (ed.) *Persistent Spectral Hole-Burning: Science and Application* (Springer, Berlin, 1988).
10. Myers, A. B., Tchénio, P., Zgierski, M. Z. & Moerner, W. E. Vibronic spectroscopy of individual molecules in solids. *J. Phys. Chem.* **98**, 10377–10390 (1994).
11. Tchénio, P., Myers, A. B. & Moerner, W. E. Vibrational analysis of the dispersed fluorescence from single molecules of terryene in polyethylene. *Chem. Phys. Lett.* **213**, 325–332 (1993).
12. Kummer, S. *et al.* Absorption, excitation and emission spectroscopy of terryene in p -terphenyl: bulk measurements and single molecule studies. *J. Chem. Phys.* (submitted).
13. Baudour, J. L., Delugeard, Y. & Cailleau, H. Structure cristalline de la phase basse température du p -terphényle à 113 K. *Acta Crystallogr. B* **32**, 150–154 (1976).
14. Reilly, P. D. & Skinner, J. L. Spectral diffusion of individual pentacene molecules in a p -terphenyl crystal: stochastic theoretical model and analysis of experimental data. *J. Chem. Phys.* **102**, 1540–1552 (1995).
15. Ambrose, W. P. & Moerner, W. E. Fluorescence spectroscopy and spectral diffusion of single impurity molecules in a crystal. *Nature* **349**, 225–227 (1991).

Acknowledgements. We thank C. Krysch for discussions. This work was supported by the Deutsche Forschungsgemeinschaft.

Correspondence should be addressed to T.B.

Control of atmospheric export of dust from North Africa by the North Atlantic Oscillation

Cyril Moulin^{*}, Claude E. Lambert[†], François Dulac[‡] & Uri Dayan[‡]

^{*}Laboratoire de Modélisation du Climat et de l'Environnement, CEA, 91191 Gif-sur-Yvette cedex, France

[†]Centre des Faibles Radioactivités, CNRS-CEA, 91198 Gif-sur-Yvette cedex, France

[‡]Environmental and Risk Assessment Section, SNRC, 81800 Yavne, Israël

All year long, massive airborne plumes of desert dust from the Sahara and surrounding regions are exported to the North Atlantic Ocean¹ and the Mediterranean Sea². The mass of African dust transported in the atmosphere is large—about one billion tonnes per year (ref. 3)—and it has been suggested that the wind-blown dusts have a substantial influence on the regional radiative budget^{4–6}. Here we use daily satellite observations of airborne dusts⁷ to obtain an 11-year regional-scale analysis of dust transport out of Africa. The substantial seasonal variability over the Atlantic Ocean and Mediterranean Sea can be explained by the synoptic meteorology. Interannual variations in dust transport are similar over both regions, and are well correlated with the climatic variability defined by the North Atlantic Oscillation⁸. This large-scale climatic control on the dust export is effected through changes in precipitation and atmospheric circulation over the regions of dust mobilization and transport. Such natural variability is so large that it is difficult to resolve any anthropogenic influences on atmospheric dust loads, such as those due to desertification or land-use changes. It seems likely that the North Atlantic Oscillation will also affect the distribution—and radiative influence—of other aerosols.

Arid regions such as the Sahara and semi-arid regions such as North Africa or Sahel are prone to mobilization of dust particles which are transported over the Mediterranean or the Atlantic for long distances¹. Much of these dust aerosols is transported westward under the influence of trade winds whereas northward transport over the Mediterranean is linked to the presence of cyclones. The seasonal pattern of African dust transport deduced from Meteosat satellite images is shown in Fig. 1. Over the Atlantic, the zone of maximum dust transport shifts northwards from $\sim 5^\circ\text{N}$ during winter to $\sim 20^\circ\text{N}$ during summer. This seasonal migration is associated with the latitudinal movement of the large-scale circulation, including that of the Intertropical Convergence Zone. During winter, the desert aerosols are transported across the Atlantic towards the northeastern coast of South America⁹. During summer, their mean route is more northward, and they reach the

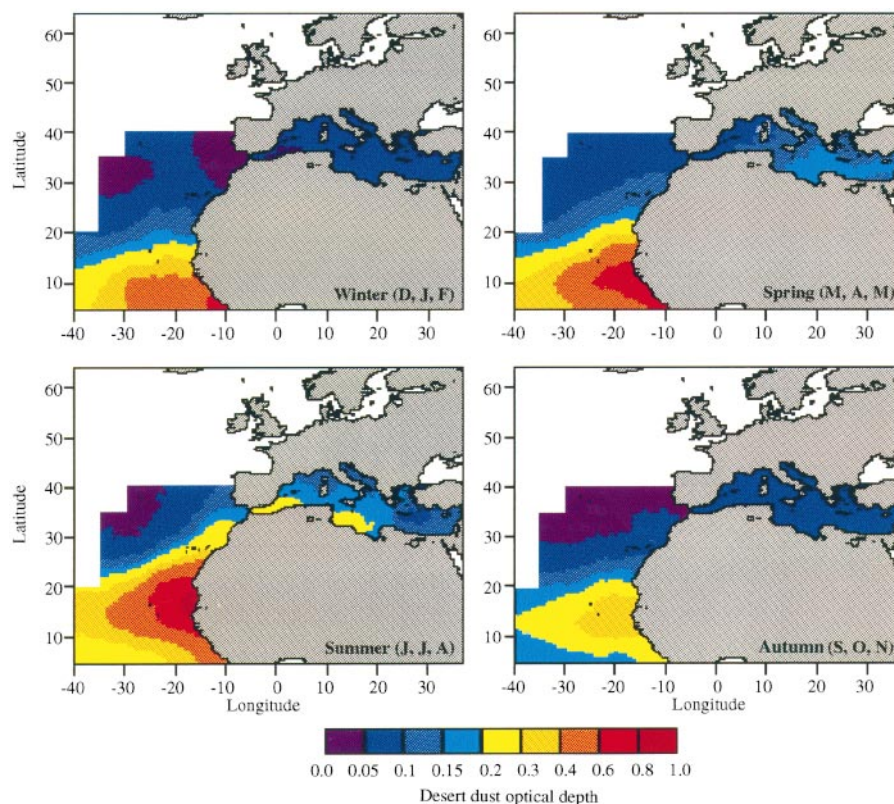


Figure 1 Seasonal maps of the desert dust optical depth (at a wavelength of $0.55\ \mu\text{m}$) over the Atlantic Ocean and the Mediterranean Sea, obtained by averaging 4,000 daily Meteosat images of the solar (VIS) channel from 1984 to 1994. Total surfaces of the Mediterranean and Atlantic zones of $2.671 \times 10^6\ \text{km}^2$ and $9.518 \times 10^6\ \text{km}^2$, respectively. The analysed zone over the Atlantic is limited to regions offering good precision due to favourable viewing geometry on the images taken at 11:45 UT, but it should be noted that dust transport extend more widely over the Atlantic south and west of this operational zone. Low-resolution images (ISCCP-B2 format; European Space Agency, Darmstadt, Germany) are composed of 416 lines of 416 pixels with an apparent resolution of $30 \times 30\ \text{km}$ at nadir. During the period, different platforms were used: Meteosat 2 from 1984 to mid-1988, Meteosat 3 from mid-1988 to mid-1989, Meteosat 4 from mid-1989 to

early 1994, and Meteosat 5 until the end of 1994. We took into account a detailed calibration of the different sensors²⁰. The method used to analyse Meteosat images is that of Dulac *et al.*²¹, adapted for multi-year monitoring of daily African dust load over the Mediterranean and the North Atlantic⁷. For each clear-sky marine pixel, the optical depth of desert dust at $0.55\ \mu\text{m}$ has been retrieved using a radiative transfer model²², in order to equate the measured and simulated radiances at the satellite level. The accuracy of the dust optical depth on one clear-sky pixel was then estimated to be within $\pm 25\%$ (ref. 23). To avoid any climatological bias due to the heterogeneous distribution of clouds²⁴, the complete optical depth field was reconstructed for each image using an interpolation for every cloudy pixel⁷.

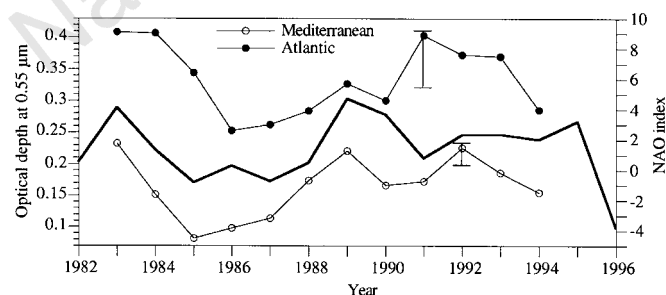


Figure 2 Comparison between the North Atlantic Oscillation (NAO) index (bold continuous line; J. W. Hurrell, personal communication) and the mean optical depth of desert dust in summer from 1983 to 1994 over the whole Mediterranean (open circles) and the Atlantic zone defined in Fig. 1 (filled circles). The summer season was chosen because it enables us to extend the data set to 1983; the Meteosat ISCCP-B2 archive started only in June 1983. Annual means would show similar variations as in summer. Each data point summarizes 0.5×10^6 pixels for the Mediterranean and 1.4×10^6 pixels for the Atlantic. Although we corrected our estimates of the dust optical depth for the contribution of Pinatubo sulphates in the stratosphere, potential overestimations remain, mainly for optical depths in summer 1991 over the Atlantic and in summer 1992 over the Mediterranean⁷. This is shown as error bars.

Caribbean Sea¹. Our data set for the Atlantic complements previous findings using a five-year climatology of dust transport occurrence based on Meteosat images⁴, and a four-year average of Advanced Very High Resolution Radiometer marine aerosol optical depth data¹⁰. Over the Mediterranean, the seasonal patterns of African dust transport show a maximum desert dust load in the central and eastern basins during spring, and in the central and western basins during summer (Fig. 1). This shift of desert dust transport from the east to the west of the Mediterranean is in good agreement with the climatology of Mediterranean cyclones^{11,12}. Indeed, spring and early summer constitute the favourable period for the *sharav* cyclones which tend to move along the north African coast and to cross the Mediterranean towards the north between Tunisia and Egypt. During summer, the Saharan depression combined with the semi-permanent ridge over Libya act in concert to transport dust over the western and central basins. At the end of summer, most cyclones originate from the Balearic region and their main route is northeast toward Corsica and Italy². This observed mean pattern in Fig. 1 is repeated from year to year for the Atlantic with no major change except in the intensity of the dust export. By contrast, the inter-annual change in the intensity of dust export over the Mediterranean is associated with large differences in the regional localization of dust maxima.

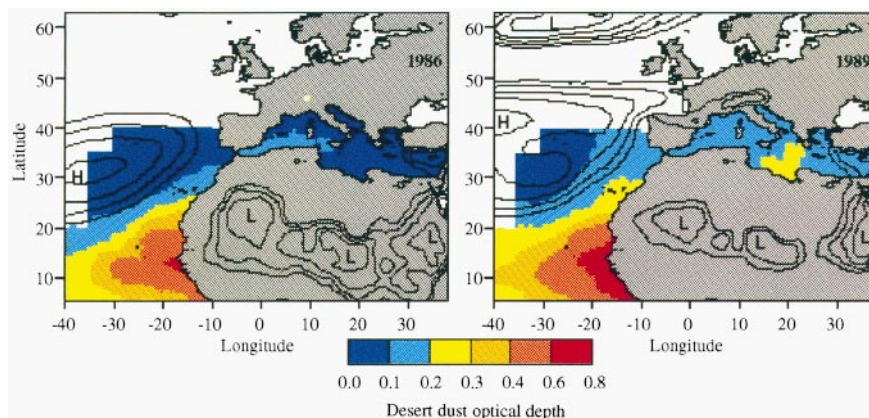


Figure 3 Maps of the desert dust optical depth (averaged from March to August) over the Mediterranean and the Atlantic for a dust-poor year (1986; left) and a dust-rich year (1989; right). The coincident mean sea level pressure fields (averaged from March to August) are drawn from ECMWF data¹⁴. For clarity, only the lowest and highest pressure contours are shown, respectively decreasing from

1,010 mbar in steps of 1 mbar for lows and increasing from 1,020 mbar in steps of 1 mbar for highs. Mean optical depth over the Atlantic is 0.17 in 1986 and 0.24 in 1989. Over the whole Mediterranean, the mean optical depth is 0.06 in 1986 and 0.13 in 1989.

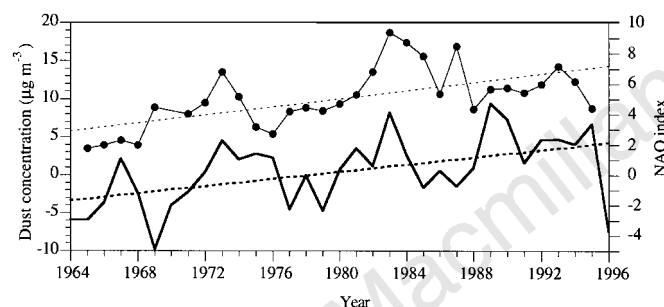


Figure 4 Comparison of the North Atlantic Oscillation (NAO) index (bold continuous line; J. W. Hurrell, personal communication) between 1964 and 1996 with the annual mean of desert dust surface concentrations at Barbados, West Indies, between 1965 and 1995 (filled circles; J. M. Prospero, personal communication). Dotted lines show the regression line for each variable. The NAO upward trend began only 30 years ago⁹.

Despite the differences in the source regions and transport processes of dust for the Mediterranean and for the Atlantic, we observed a significant year-to-year correlation of the intensity of dust export over these two oceans. Figure 2 shows simultaneous variations of the mean dust load in summer over the Atlantic and the Mediterranean during the 11-year period with maxima in 1983, 1989 and 1991–92 ($r = 0.50$, $p = 0.097$). These similarities are the sign that large-scale forces are constraining the dust export to both the Atlantic and the Mediterranean. Precipitation is one of the most efficient processes that may induce interannual variability in the transport of African dust. Precipitation is highly variable from year to year and may affect the atmospheric dust cycle through both a change in soil moisture in source regions and by washout during transport. However, other meteorological factors, such as large-scale dynamical features of the atmosphere, can also contribute to this year-to-year variability¹.

The North Atlantic Oscillation (NAO) appears to exert a strong influence on the large-scale variations of both the atmospheric circulation and the hydrological cycle in the Northern Hemisphere⁸. An index of the NAO is defined by Hurrell⁸ from the difference between normalized winter sea-level atmospheric pressures between Lisbon, Portugal, and Stykkisholmur, Iceland. Whereas this oscillation is more pronounced during winter, it is present throughout the

year. Winters with high NAO indices are characterized by a deepening of the Icelandic low associated with a stronger Azores anticyclone. The related changes in the large-scale meteorological situation induce a northward shift of the North Atlantic westerlies which provide much of the atmospheric moisture to northern Africa and Europe. In the case of high NAO index, this shift yields drier conditions over southern Europe, the Mediterranean Sea and northern Africa, together with changes in the storm tracks in these regions^{8,13}. During low-NAO years, precipitation is likely to be greater over the Mediterranean and large areas of North Africa, limiting the intensity of both dust uptake and transport. The NAO index is plotted with our dust load data in Fig. 2. It covaries with desert dust transport: the correlation coefficient between NAO and dust load is 0.66 for the Mediterranean ($p = 0.027$) and 0.49 for the Atlantic ($p = 0.124$).

Figure 3 illustrates the effect of the NAO on both pattern and intensity of the transport of African dust. We compare 6-month means (March–August) of the desert dust optical depth and of the sea-level pressure¹⁴ over the Mediterranean and the northern Atlantic for two contrasting years: 1986 characterized by a low NAO index (0.28), and 1989 with a high NAO index (4.73). The spatial extent of the desert dust load over the Atlantic is about the same, but the mean optical depth is significantly different (0.17 in 1986, 0.24 in 1989). Over the Mediterranean, both transport pattern and intensity (mean optical depth of 0.06 in 1986 and of 0.13 in 1989) are different, with a particularly strong desert dust load over the central basin in 1989, on the route of the *sharav* cyclones. The difference in the NAO indices is shown by the location and strength of the main meteorological centres. Compared to 1986, a deep Icelandic low centred around 60°N is clearly shown in 1989. This comparison illustrates the role of the NAO in controlling the desert dust transport. In addition to the rainfall deficit, the link between NAO and synoptic meteorology (for example, cyclone tracks) may be of primary importance in the Mediterranean. Since early June 1996, we have used on-line Meteosat images to monitor African dust transport to the Mediterranean; there has been an unusual lack of summer dust outbreaks in this region, an observation that is consistent with one of the lowest NAO indices this century (-3.88 ; J. W. Hurrell, personal communication).

To extend our satellite-derived data set, we used surface concentration measurements of mineral aerosol at Barbados, West Indies (J. M. Prospero, personal communication). These data are available between 1965 and 1995, as shown in Fig. 4. Compared to our large-

scale data set, these data derived from a single remote site can be more influenced by local meteorological conditions. Despite this potential perturbation, the annual mean of dust concentrations at Barbados generally follows the same variations as the NAO index ($r = 0.43$, $p = 0.018$). The long-term increase of the desert dust load¹⁵ also corresponds to the upward trend of the NAO since the early 1970s. Both upward trends shown in Fig. 4 are statistically significant. Both interannual variability and decadal increase of the African dust export, as observed from Meteosat images and mineral aerosol at Barbados, are thus apparently controlled by climatic conditions expressed by the NAO index. This climatic effect prevents us from establishing a simple relation between the increasing trend in dust load and a direct anthropogenic forcing such as the desertification due to human activity¹⁶: although a direct link between anthropogenic desertification and dust transport is expected^{17,18}, it will be difficult to deconvolve it from the general climatic oscillation. However, it is likely that the NAO 'see-saw' also affects the concentrations of anthropogenic aerosols such as sulphates from pollution and carbonaceous aerosols from biomass burning. Indeed, these aerosols are certainly also strongly sensitive to the NAO, as transport processes and washout affect all particle sizes. It is thus likely that anthropogenic aerosol radiative effects exhibit strong interannual and decadal variabilities which should be taken into account by modelling efforts attempting to predict future climate change due to human activity¹⁹. □

Received 21 October 1996; accepted 21 April 1997.

- Prospero, J. M. in *Particle Flux in the Ocean* (eds Schäfer, P., Ittekkot, V., Honjo, S. & Depetris, P. J.) 19–52 (Wiley, New York, 1996).
- Bergametti, G. et al. in *Paleoclimatology and Paleometeorology: Modern and Past Patterns of Global Atmospheric Transport* (eds Leinen, M. & Santhorn, M.) 227–251 (Kluwer, Dordrecht, 1989).
- D'Almeida, G. A. A model for Saharan dust transport. *J. Clim. Appl. Meteorol.* **24**, 903–916 (1986).
- Jankovik, I. & Tantré, D. Satellite climatology of Saharan dust outbreaks: Method and preliminary results. *J. Clim.* **5**, 646–656 (1992).
- Tegen, I., Lacis, A. A. & Fung, I. The influence on climate forcing of mineral aerosols from disturbed soils. *Nature* **380**, 419–422 (1996).
- Li, X., Maring, H., Savoie, D., Voss, K. & Prospero, J. M. Dominance of mineral dust in aerosol light scattering in the North Atlantic trade winds. *Nature* **380**, 416–419 (1996).
- Moulin, C., Guillard, F., Dulac, F. & Lambert, C. E. Long-term daily monitoring of Saharan dust load over marine areas using Meteosat ISCCP-B2 data. 1: Methodology and preliminary results for 1983–1994 in the Western Mediterranean. *J. Geophys. Res.* (in the press).
- Hurrell, J. W. Decadal trend in the North Atlantic Oscillation: Regional temperatures and precipitations. *Science* **269**, 676–679 (1995).
- Swap, R., Garstang, M., Greco, S., Talbot, R. & Källberg, P. Saharan dust in the Amazon Basin. *Tellus* **44B**, 133–149 (1992).
- Swap, R., Ulanski, S., Cobbett, M. & Garstang, M. Temporal and spatial characteristics of Saharan dust outbreaks. *J. Geophys. Res.* **101**, 4205–4220 (1996).
- Alpert, P., Neeman, B. U. & Shay-el, Y. Climatological analysis of Mediterranean cyclones using ECMWF data. *Tellus* **42A**, 65–77 (1990).
- Dayan, U., Heffter, J., Miller, J. & Gutman, G. Dust intrusion events into the Mediterranean basin. *J. Appl. Meteorol.* **30**, 1185–1198 (1991).
- Pittalwala, I. I. & Hameed, S. Simulation of the North Atlantic Oscillation in a general circulation model. *Geophys. Res. Lett.* **18**, 841–844 (1991).
- Siefridt, L. & Barnier, B. *Archive AVISO Vent-Flux: Analyse de Surface du ECMWF Accessibles au CCVR* (CCVR, Grenoble, 1994).
- Prospero, J. M. & Nees, R. T. Impact of the North African drought and El Niño on the mineral dust in the Barbado trade winds. *Nature* **320**, 735–738 (1986).
- Andreae, M. O. Raising dust in the greenhouse. *Nature* **380**, 389–390 (1996).
- Sokolik, I. N. & Toon, O. B. Direct radiative forcing by anthropogenic airborne mineral aerosols. *Nature* **381**, 681–683 (1996).
- Tucker, C. J., Newcomb, W. W. & Dregne, H. E. AVHRR data sets for determination of desert spatial extent. *Int. J. Remote Sens.* **15**, 3547–3565 (1994).
- Houghton, J. T. et al. (eds) *Climate Change 1995: The Science of Climate Change* (Cambridge Univ. Press, 1996).
- Moulin, C., Lambert, C. E., Poitou, J. & Dulac, F. Long-term (1983–1984) calibration of the Meteosat solar (VIS) channel using desert and ocean targets. *Int. J. Remote Sens.* **17**, 1183–1200 (1996).
- Dulac, F. et al. Assessment of the African airborne dust mass over the Western Mediterranean using Meteosat data. *J. Geophys. Res.* **97**, 2489–2506 (1992).
- Tantré, D. et al. Description of a computer code to simulate the satellite signal in the solar spectrum: The 5S code. *Int. J. Remote Sens.* **11**, 659–668 (1990).
- Moulin, C. et al. Long-term daily monitoring of Saharan dust load over marine areas using Meteosat ISCCP-B2 data. 2: Accuracy of the method and validation using Sun photometer measurements. *J. Geophys. Res.* (in the press).
- Dayan, U., Heffter, J. L. & Miller, J. M. *Meteorological and Climatological data from Surface and Upper Air Measurements for the Assessment of Atmospheric Transport and Deposition of Pollutants in the Mediterranean Basin* (UNEP, Athens, 1989).

Acknowledgements. We thank J. W. Hurrell and J. M. Prospero for providing us with their data, and for their comments; Y. Balkanski for facilitating the access to the AVISO archive; V. Masson, J. Orr and B. Ziv for discussions about climatic variability; and W. Guelle and X. Schneider for their utility computer codes. This work was supported by the CEA and the CNRS.

Correspondence and requests for materials should be addressed to F.D. (e-mail: dulac@cea.fr).

Perovskite as a possible sink for ferric iron in the lower mantle

Catherine McCammon

Bayerisches Geoinstitut, Universität Bayreuth, D-95440 Bayreuth, Germany

The lower mantle constitutes more than half the Earth's interior by volume, and is believed to consist predominantly of (Mg,Fe)SiO₃ perovskite with up to approximately 20% (Mg,Fe)O. In the system FeO–MgO–SiO₂, iron partitions preferentially into (Mg,Fe)O relative to the perovskite phase and has been believed to be nearly all in the form of Fe²⁺ on the basis of experiments in the MgO–FeO–SiO₂ system^{1–4}. Here, however, we present a Mössbauer study of (Mg,Fe)SiO₃ perovskite containing 3.3 mol% Al₂O₃, which shows that approximately 50% of the iron is Fe³⁺. These results, combined with evidence from other experiments, suggest that the proportion of iron present as Fe³⁺ in the lower-mantle perovskite phase is probably much higher than is currently believed. Because the oxidation state of iron in the perovskite phase affects the electrostatic charge balance and equilibrium defect concentration, the presence of Fe³⁺ is likely to significantly affect physical and chemical properties of the lower mantle such as sub- and super-solidus phase relations, transport properties, mechanical behaviour, trace-element partitioning and the concentration of species such as hydroxide ions.

Natural orthopyroxene from San Carlos, Arizona was packed into a rhenium capsule with a small amount of ReO₂ to constrain oxygen fugacity conditions during the run, and transformed to a perovskite phase at 25 GPa and 1600 °C for 2 h using the 1,200-ton multianvil press with a LaCrO₃ heater assembly at the Bayerisches Geoinstitut. Temperature was initially measured with a W₇₅Re₂₅/W₉₇Re₃ thermocouple, but because of thermocouple failure during heating, the final temperature was estimated based on a calibrated power curve derived from three previous experiments using the same assembly at the same pressure and temperature conditions. Previous results suggest the temperature uncertainty in this case to be approximately ±50 °C; however it could be higher. As the goal was only to synthesize an Al-containing perovskite phase, the temperature uncertainty does not affect the conclusions of this study. To avoid back transformation or amorphization, the sample was cooled to 77 K and crushed in a pellet press that was also precooled with liquid nitrogen. Approximately 3 mg of crushed sample was recovered and mounted within a 3-mm-diameter spot on a plastic foil for powder X-ray diffraction and Mössbauer analysis. Transmission Mössbauer spectra were recorded on a triangular velocity constant acceleration Mössbauer spectrometer with a nominal 50 mCi ⁵⁷Co source in a 6-μm Rh matrix. Spectra were recorded at 80 K using a continuous-flow cryostat with the sample in nitrogen vapour. The velocity scale was calibrated with respect to 25-μm Fe foil using the positions certified for National Bureau of Standards standard reference material No. 1541; linewidths for Fe of 0.28 mm s⁻¹ were obtained at 298 K. The spectra were fitted to lorentzian lineshapes using the fitting program NORMOS written by R. A. Brand (distributed by Wissenschaftlich Elektronik GmbH, Germany). The thickness of the Mössbauer sample was ~4 mg Fe cm⁻².

Microprobe analyses of the run product gave results identical to those of the starting material within experimental error (Table 1). Rhenium was analysed for, but not found in the perovskite phase, and no Fe was found in the Re metal. X-ray powder diffraction data indicated that the sample consists of a single perovskite phase with trace amounts of stishovite and Re metal.

Table 1 Microprobe analyses of Al-perovskite phase

Oxide	wt%	Cation/3 oxygen*
SiO ₂	57.02	Si ⁴⁺ 0.968(12)
TiO ₂	0.04	Ti ⁴⁺ 0.000(1)
Al ₂ O ₃	3.29	Al ³⁺ 0.065(1)
Cr ₂ O ₃	0.59	Cr ³⁺ 0.008(1)
FeO	5.55	Fe ²⁺ 0.040(7)
		Fe ³⁺ 0.040(5)
MnO	0.00	Mn ²⁺ 0.002(1)
NiO	0.12	Ni ²⁺ 0.001(1)
MgO	33.90	Mg ²⁺ 0.854(11)
CaO	0.88	Ca ²⁺ 0.016(1)
Na ₂ O	0.04	Na ⁺ 0.001(1)
K ₂ O	0.01	K ⁺ 0.000(1)
Total	101.52	1.995

* Numbers in parentheses are standard deviations calculated on the basis of 37 analyses on four different grains. Cation proportions are calculated on the basis of three oxygens per formula unit.

Using Si as an internal calibration standard, the following refined cell parameters were determined for the perovskite phase: $a = 4.7917(4) \text{ \AA}$, $b = 4.9459(4) \text{ \AA}$, $c = 6.9263(7) \text{ \AA}$ and $V = 164.15(2) \text{ \AA}^3$. The volume is 0.4% higher than predicted by the empirical relation of O'Neill and Jeanloz⁵, but the deviation falls within the range observed for other (Mg,Fe,Al)(Si,Al)O₃ perovskites by other investigators (Fig. 6 in ref. 5).

To provide a comparison, a sample of Al-free Mg_{0.94}Fe_{0.06}SiO₃ perovskite was synthesised from synthetic orthopyroxene in the multianvil press where conditions were nearly identical to the above synthesis (Re capsule, LaCrO₃ furnace, 24 GPa, 1,650 °C, 30 min)⁶. The room-temperature Mössbauer spectrum of the sample is illustrated in Fig. 1a, and shows the presence of Fe²⁺, Fe³⁺ and rapid electron transfer between Fe²⁺ and Fe³⁺. Hyperfine parameters are similar to other values reported for Al-free (Mg,Fe)SiO₃ perovskite^{7,8}. The relative amount of Fe³⁺ can be determined from the relative areas corrected for differences in recoil-free fraction⁹ and is found to be $16 \pm 3\%$ for this sample. This is similar to the amount found by Fei *et al.*⁸ in perovskites synthesised under similar conditions, and is close to the currently believed maximum plausible Fe³⁺/ΣFe value for the lower-mantle perovskite phase as the Re–ReO₂ buffer is relatively oxidizing (the buffer curve lies approximately midway between Ni–NiO and Fe₃O₄–Fe₂O₃ at 1 bar; ref. 10).

The room-temperature Mössbauer spectrum of the Al-containing (Mg,Fe)SiO₃ perovskite phase from the present study is illustrated in Fig. 1b, and shows significant Fe³⁺. Table 2 lists the hyperfine parameters and relative areas of the component sub-spectra derived from Mössbauer spectra recorded at 298 and 80 K. By applying the same corrections to the relative areas for recoil-free fraction that were used above, the value Fe³⁺/ΣFe = $50 \pm 5\%$ is obtained. The orthopyroxene starting material contained no Fe³⁺ that could be detected using Mössbauer spectroscopy.

The presence of Al stabilizes Fe³⁺ in the perovskite structure. This possibility has already been mentioned by McCammon¹¹ to explain results of Kesson *et al.*¹² who showed that single-phase perovskite can be synthesised from a starting material of the composition almandine₇₅pyrope₂₅. It was also suggested by Wood and Rubie¹³ as a possibility to account for the reduced Fe–Mg partition coefficient between (Mg,Fe)O and (Mg,Fe)SiO₃ perovskite when the latter phase contains Al. In this case the partition coefficient assumes that all iron is Fe²⁺, but if a significant amount of Fe in the perovskite phase is Fe³⁺ then the partition coefficient is reduced.

It is generally understood that Fe²⁺ occupies the large distorted site in the perovskite structure (ref. 14 and references therein), but the location of Fe³⁺ has been more difficult to determine owing to its low concentration. Previous results suggest that Fe³⁺ preferentially occupies the octahedral site^{6,8,9}. The data in the present study strongly suggest that Fe³⁺ occupies both sites, because (1) the centre shift corresponding to Fe³⁺ in the Al-perovskite phase

Table 2 Hyperfine parameters of Al-perovskite phase

	293 K		80 K	
	Fe ²⁺	Fe ³⁺	Fe ²⁺	Fe ³⁺
Centre shift* (mm s ⁻¹)	1.10(3)†	0.46(1)	1.27(2)†	0.60(1)
Quadrupole splitting (mm s ⁻¹)	2.02(5)†	0.85(2)	2.27(4)†	0.83(2)
Linewidth (mm s ⁻¹)	0.52(5)†	0.48(2)	0.43(3)†	0.44(2)
Relative area (%)	40	60	47	53

Numbers in parentheses refer to the standard deviation of the last digit.

* Relative to α-Fe.

† Weighted mean value.

(0.46 mm s⁻¹) is significantly higher than the value expected for octahedral Fe³⁺ (ref. 11), and (2) a simple calculation using the cation data in Table 1 shows Si⁴⁺ + Fe³⁺ to be greater than one. The substitution Mg²⁺ + Si⁴⁺ → Al³⁺ + Fe³⁺ is therefore a likely mechanism to stabilize Fe³⁺ and maintain electrostatic charge balance in the crystal structure.

Fe³⁺ is likely to be concentrated in the perovskite phase relative to (Mg,Fe)O in the lower mantle, because (1) high-pressure experiments on a pyrolite mantle composition have shown that aluminium is concentrated in the perovskite phase¹⁵ and (2) high-pressure experiments on (Mg,Fe)O suggest that the solubility of Fe³⁺ decreases dramatically at high pressure¹⁶. For example, the (Mg,Fe)O–Fe(Mg,Fe)₂O₄ boundary corresponds to maximum solubility of Fe³⁺ in Mg_{0.8}Fe_{0.2}O at 1,000 °C and 1 bar of Fe³⁺/ΣFe = 44% (ref. 17). The Re–ReO₂ buffer is more oxidizing than this boundary at these conditions. However, at 1,000 °C and 18 GPa, multianvil experiments performed on oxidized Mg_{0.8}Fe_{0.2}O + a trace of ReO₂ in an Re capsule showed that the amount of Fe³⁺ is reduced to only a few per cent Fe³⁺/ΣFe as measured by Mössbauer spectroscopy. During the high-pressure experiment, the black starting material was converted to pale yellow with small black particles which are believed to be a spinel phase. Fe³⁺ is stabilized in the spinel phase relative to (Mg,Fe)O probably as the result of a high-pressure phase transition in the system Fe₃O₄–MgFe₂O₄ (C.McC., manuscript in preparation).

The amount of Fe³⁺ in the lower-mantle perovskite phase is probably a function of both Al₂O₃ content and oxygen fugacity. Experiments are currently underway to investigate the relation between these variables; preliminary results show that Fe³⁺ is equally high in (Mg,Fe,Al)(Si,Al)O₃ perovskites synthesized under reducing conditions (S. Lauterbach, C.McC. and F. Seifert, unpublished data). More data is required, however, before a full evaluation of the relation between these variables can be made.

Wood and Rubie¹³ observed that Fe–Mg partitioning is drastically altered by the addition of 4–5 wt% Al₂O₃, resulting in an essentially equal partitioning of iron between the perovskite and (Mg,Fe)O phases. If the Fe²⁺–Mg partition coefficient between the perovskite phase and (Mg,Fe)O is assumed to be the same in both Al-free systems and Al-containing systems, implying that the difference is due to Fe³⁺, results from Wood and Rubie's experiments¹³ give values of 70–90% Fe³⁺/ΣFe. Partition coefficients from experiments using different capsule materials were similar, implying that differences in oxygen fugacity had little effect on the results. These authors were not able to measure Fe³⁺, however, so the effect of oxygen fugacity on the Fe³⁺ content of the perovskite phase cannot be directly evaluated.

We are not yet able to estimate the amount of Fe³⁺ in the lower-mantle perovskite phase; but the strong stabilizing effect of Al³⁺ on Fe³⁺ in the perovskite phase combined with the evidence discussed

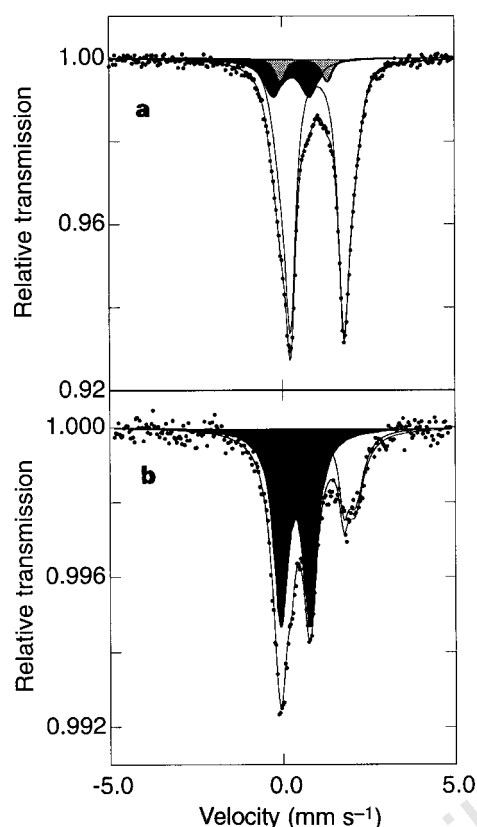


Figure 1 Mössbauer spectra of (Mg,Fe)SiO₃ perovskite: **a**, Al-free; **b**, containing 3.3 wt% Al₂O₃. Fe³⁺ absorption is shown as black in the spectra and peaks due to Fe²⁺–Fe³⁺ electron transfer are shaded grey. Fe²⁺ absorption is modelled by two doublets which are thought to arise from differences in next-nearest neighbour environments⁸. Note the dramatic increase in relative Fe³⁺ content of the perovskite in the presence of Al.

above strongly suggests that it is significantly higher than currently believed.

Replacement of Fe²⁺ with Fe³⁺ in the perovskite phase is likely to affect significantly physical and chemical properties of the lower mantle such as sub- and super-solidus phase relations, transport properties, mechanical behaviour, trace-element partitioning and the concentration of species such as OH[–]. For example, comparison of lower-mantle electrical conductivity models with laboratory measurements of lower-mantle phases in the system MgO–FeO–SiO₂ have been controversial (ref. 18 and references therein). Based on the present results, however, samples containing Al would be more representative of the lower mantle. Electrical-conductivity measurements of natural orthopyroxene show that addition of small amounts of Al₂O₃ increases electrical conductivity significantly, and that conductivity is relatively independent of oxygen fugacity¹⁹. By analogy with pyroxene, it is likely that electrical conductivity of (Mg,Fe,Al)SiO₃ perovskite would also be significantly higher than for the Al-free phase, as the conductivity mechanism is believed to involve Fe³⁺ (ref. 20). Laboratory measurements of electrical conductivity on Al-containing (Mg,Fe)SiO₃ perovskite are therefore required before a realistic comparison with bulk geophysical data can be made. □

Received 5 December 1996; accepted 22 April 1997.

1. Ito, E., Takahashi, E. & Matsui, Y. The mineralogy and chemistry of the lower mantle: an implication of the ultrahigh-pressure phase relations in the system MgO–FeO–SiO₂. *Earth Planet. Sci. Lett.* **67**, 238–248 (1984).
2. Guyot, F., Madon, M., Peyronneau, J. & Poirier, J. P. X-ray microanalysis of high-pressure/high-temperature phases synthesized from natural olivine in a diamond anvil cell. *Earth Planet. Sci. Lett.* **90**, 52–64 (1988).
3. Fei, Y., Mao, H. K. & Mysen, B. O. Experimental determination of element partitioning and

calculation of phase relations in the MgO–FeO–SiO₂ system at high pressure and high temperature. *J. Geophys. Res.* **96**, 2157–2169 (1991).

4. Kesson, S. E. & Fitz Gerald, J. D. Partitioning of MgO, FeO, NiO, MnO and Cr₂O₃ between magnesian silicate perovskite and magnesio-wüstite: Implications for the origin of inclusions in diamond and the composition of the lower mantle. *Earth Planet. Sci. Lett.* **111**, 229–240 (1991).
5. O'Neill, B. & Jeanloz, R. MgSiO₃–FeSiO₃–Al₂O₃ in the Earth's lower mantle: Perovskite and garnet at 1,200 km depth. *J. Geophys. Res.* **99**, 19901–19915 (1994).
6. Keppler, H., McCammon, C. A. & Rubie, D. C. Crystal field and charge transfer spectra of (Mg,Fe)SiO₃ perovskite. *Am. Mineral.* **79**, 1215–1218 (1994).
7. McCammon, C. A., Rubie, D. C., Ross, C. R., Seifert, F. & O'Neill, H. S. C. Mössbauer spectra of ⁵⁷Fe_{0.05}Mg_{0.95}SiO₃ perovskite at 80 and 298 K. *Am. Mineral.* **77**, 894–897 (1992).
8. Fei, Y., Virgo, D., Mysen, B. O., Wang, Y. & Mao, H. K. Temperature dependent electron delocalization in (Mg,Fe)SiO₃ perovskite. *Am. Mineral.* **79**, 826–837 (1994).
9. McCammon, C. A. The crystal chemistry of ferric iron in Fe_{0.05}Mg_{0.95}SiO₃ as determined by Mössbauer spectroscopy. *Phys. Chem. Miner.* (submitted).
10. Pownceby, M. I. & O'Neill, H. S. C. Thermodynamic data from redox reactions at high temperatures. IV. Calibration of the Re–ReO₂ oxygen buffer from EMF and NiO + NiH₂–Pd redox sensor measurements. *Contrib. Mineral. Petrol.* **118**, 130–137 (1994).
11. McCammon, C. A. Crystal chemistry of iron-containing perovskites. *Phase Transitions* **58**, 1–26 (1996).
12. Kesson, S. E., Fitz Gerald, J. D., Shelley, J. M. G. & Withers, R. L. Phase relations, structure and crystal chemistry of some aluminous silicate perovskites. *Earth Planet. Sci. Lett.* **134**, 187–201 (1995).
13. Wood, B. J. & Rubie, D. C. The effect of alumina on phase transformations at the 660-kilometer discontinuity from Fe–Mg partitioning experiments. *Science* **273**, 1522–1524 (1996).
14. Farges, F., Guyot, F., Andrault, D. & Wang, Y. Local structure around Fe in Mg_{0.9}Fe_{0.1}SiO₃ perovskite: An X-ray absorption spectroscopy study at Fe–K edge. *Eur. J. Mineral.* **6**, 303–312 (1994).
15. Irifune, T. Absence of an aluminous phase in the upper part of the Earth's lower mantle. *Nature* **370**, 131–133 (1994).
16. McCammon, C. A., Harris, J. W., Harte, B. & Hutchison, M. T. Partitioning of ferric iron between lower mantle phases: Results from natural and synthetic samples. *J. Conf. Abstr.* **1**, 390 (1996).
17. Speidel, D. H. Phase equilibria in the system MgO–FeO–Fe₂O₃: The 1300 °C isothermal section and extrapolations to other temperatures. *J. Am. Ceram. Soc.* **50**, 243–248 (1967).
18. Duba, A. G. & Wanamaker, B. J. DAC measurement of perovskite conductivity and implications for the distribution of mineral phases in the lower mantle. *Geophys. Res. Lett.* **21**, 1643–1646 (1994).
19. Huebner, J. S., Duba, A. & Wiggins, L. B. Electrical conductivity of pyroxene which contains trivalent cations: Laboratory measurements and the lunar temperature profile. *J. Geophys. Res.* **84**, 4652–4656 (1979).
20. Poirier, J. P. & Peyronneau, J. in *High-Pressure Research: Application to Earth and Planetary Sciences* (eds Syono, Y. & Manghnani, M. H.) 77–87 (Terra Scientific/American Geophysical Union, Tokyo/Washington DC, 1992).

Acknowledgements. The manuscript was improved through discussions with A. Duba, B. Harte, M. Hutchison, S. Lauterbach, J. Peyronneau, J.-P. Poirier, F. Seifert and F. Viscoekas. The sample of natural orthopyroxene was provided by J. Peyronneau and J.-P. Poirier, and the spectrum of the Al-perovskite phase was recorded at 80 K by S. Lauterbach. Mössbauer experiments were performed using equipment provided by F. Seifert, who was supported by Fonds der Chemischen Industrie (Germany).

Correspondence should be addressed to C.McC. (e-mail: catherine.mccammon@uni-bayreuth.de).

Luminescence dating of rock art and past environments using mud-wasp nests in northern Australia

Richard Roberts*, Grahame Walsh†, Andrew Murray‡, Jon Olley§, Rhys Jones||, Michael Morwood¶, Claudio Tuniz#, Ewan Lawson#, Michael Macphail||, Doreen Bowdery* & Ian Naumann*

* School of Earth Sciences, La Trobe University, Melbourne, Victoria 3083, Australia

† Takarakka Rock Art Research Centre, Carnarvon Gorge, Queensland 4702, Australia

‡ Nordic Laboratory for Luminescence Dating, Risø National Laboratory, DK 4000 Roskilde, Denmark

§ CSIRO Land and Water, Canberra, Australian Capital Territory 2601, Australia

|| Division of Archaeology and Natural History, RSPAS, Australian National University, Canberra, Australian Capital Territory 0200, Australia

¶ Department of Archaeology and Palaeoanthropology, University of New England, Armidale, New South Wales 2351, Australia

Physics Division, Australian Nuclear Science and Technology Organisation, Menai, New South Wales 2234, Australia

* CSIRO Entomology, Canberra, Australian Capital Territory 2601, Australia

Mud-nesting wasps are found in all of the main biogeographical regions of the world^{1–3}, and construct nests that become petrified after abandonment. Nests built by mud-dauber and potter wasps in rock shelters in northern Australia^{1,4} often overlie, and occasionally underlie, prehistoric rock paintings. Mud nests contain

pollen, spores and phytoliths from which information about local palaeovegetation can be gleaned. Here we report a new application of optical dating^{5–7}, using optically stimulated luminescence (OSL), and accelerator mass spectrometry (AMS) ¹⁴C dating of pollen⁸ to determine the ages of mud-wasp nests associated with rock paintings in the Kimberley region of Western Australia^{9,10}. Optical dating of quartz sand (including the analysis of individual grains) embedded in the mud of fossilized nests shows that some anthropomorphic paintings are more than 17,000 years old. Reconstructions of past local environments are also possible from the range of pollen and phytolith types identified. This approach should have widespread application to studies of rock-art dating and late Quaternary environmental change on continents where mud-wasps once lived and other sources of palaeo-ecological information are absent.

Optical dating provides a measure of the time since minerals, such as quartz, were last exposed to sunlight^{5–7}. Mud-nesting wasps gather surface sediments from the margins of streams and pools, further exposing any quartz grains to sunlight during collection and transport of the mud. Final exposure to the sun occurs during construction of the nest in a rock shelter. Quartz grains embedded in a nest are hidden from sunlight and will accumulate the effects of the nuclear radiation flux to which they are exposed. This radiation dose (the palaeodose) will increase with time and may be measured using optically stimulated luminescence (OSL). Mud nests constructed originally by two species of mud-dauber wasp, *Sceliphron laetum* (Smith) and *Sceliphron formosum* (Smith)¹¹, and the eumene (potter) wasp (*Abispa* spp.)^{1,4} were collected from the rain-protected ceilings and recesses of rock shelters in the northern Kimberley region of Australia. Nests were removed at night, using red-filtered (>590 nm) light, without damage to the paintings. Most of these nests were built over paintings, the relative ages of which could be estimated from a sequence of superposed motif

styles^{9,10}. Seven nests were chosen for initial dating. Three lightly cemented *S. laetum* nests (DR1, DR2 and DR6) had been built over parts of red-pigmented Wandjina (anthropomorphic) figures in one rock shelter. At another site, the residual stump of a heavily cemented, probably *S. laetum*, nest (KERC4) directly overlay the head-dress of a mulberry-coloured human figure (which, in turn, overlay a hand stencil), and this nest thickened laterally into a much larger but similarly indurated nest (KERC5). The human figure in the painting has an elongated torso, hanging arms (no decorations visible) and a narrow head from whose apex radiates a semi-circular tufted head-dress; the painting looks archaic, and may be related to the Bradshaw style^{9,10}. Two *S. laetum* nests, not obviously associated with any paintings, were also collected: an especially petrified nest (DR4) in the Wandjina rock shelter, and a 'modern' nest (KERC9, <2 years old). Nest KERC4 was no more than 5 mm thick, whereas the other nests were 20–40 mm thick.

To examine the opacity of a mud nest, the largest nest collected (DR6) was divided into seven portions, the first portion being composed of the outermost 1–3 mm of the nest mud, with successive portions consisting of mud concealed deeper within the nest; the 'core' portion had been attached to the shelter wall. Each portion was optically dated, the aim being to check that the inner portions of the nest were sufficiently light-safe to retain an OSL signal. Nest DR4 was divided into an inner core and an outer shell, while only the cores of nests DR1, DR2, KERC9 and KERC5 were dated. Quartz grains were extracted from each portion, and palaeodoses were determined using the standard multiple-aliquot additive-dose procedure¹², or the newly devised single-aliquot additive-dose and regenerative-dose protocols^{13–15}. Nest KERC4 required a different approach because of its small size: quartz grains were extracted from the entire nest and, as there were insufficient grains for standard multi-grain analysis, individual grains were dated using single-aliquot protocols. A frequency

Table 1 Dose rates, palaeodoses and optical dates for quartz grains, and AMS ¹⁴C determinations for pollen grains

Mud nest	Portion of nest	Dose rate* (mGy y ⁻¹)				AMS ¹⁴ C determinations						
		γ- and cosmic ray (1)†	γ- and cosmic ray (2)†	Nest mud β	Total dose rate	Palaeodose (Gy)‡		OSL age (y)§	Sample code	Pollen diameter (μm)	¹⁴ C activity (% M)	
KERC9	Core	0.41 ± 0.02	no data	0.56 ± 0.02	0.99 ± 0.03	0.15 ± 0.02	SA	(24)	150 ± 20	OZC361	<5	85.0 ± 1.0
										OZC362	5–10	111.1 ± 2.1
										OZC363	10–25	111.2 ± 1.1
										OZC364	45–90	111.4 ± 1.2
DR1	Core	0.61 ± 0.03	0.66 ± 0.03	0.63 ± 0.03	1.29 ± 0.04	0.13 ± 0.01	SA	(24)	100 ± 10			
DR2	Core	0.49 ± 0.03	0.52 ± 0.03	0.97 ± 0.04	1.51 ± 0.04	0.22 ± 0.01	SA	(21)	150 ± 10	OZC357	<5	88.4 ± 0.7
										OZC358	5–10	98.9 ± 3.1
										OZC359	10–25	92.4 ± 1.1
										OZC360	45–90	101.0 ± 2.7
DR6	Layer 1	0.50 ± 0.03	0.50 ± 0.03	0.87 ± 0.04	1.32 ± 0.04	0.14 ± 0.02	M	(20)	110 ± 20			
	Layer 2	0.50 ± 0.03	0.50 ± 0.03	0.87 ± 0.04	1.40 ± 0.04	0.35 ± 0.06	M	(19)	250 ± 50			
	Layer 3	0.50 ± 0.03	0.50 ± 0.03	0.87 ± 0.04	1.40 ± 0.04	0.36 ± 0.04	M	(18)	260 ± 30			
						0.41 ± 0.03	SA	(15)	290 ± 30			
	Layer 4	0.50 ± 0.03	0.50 ± 0.03	0.87 ± 0.04	1.40 ± 0.04	0.35 ± 0.04	M	(20)	250 ± 30			
	Layer 5	0.50 ± 0.03	0.50 ± 0.03	0.87 ± 0.04	1.40 ± 0.04	0.43 ± 0.08	M	(20)	310 ± 60			
	Layer 6	0.50 ± 0.03	0.50 ± 0.03	0.87 ± 0.04	1.40 ± 0.04	0.83 ± 0.12	M	(19)	590 ± 90			
	Layer 7	0.50 ± 0.03	0.50 ± 0.03	0.87 ± 0.04	1.40 ± 0.04	0.84 ± 0.17	M	(19)	600 ± 130			
						0.86 ± 0.06	SA	(3)	610 ± 50			
DR4	Outer shell	0.53 ± 0.03	no data	1.47 ± 0.07	2.05 ± 0.06	2.5 ± 0.2	M	(20)	1,240 ± 120			
	core	0.53 ± 0.03	no data	1.47 ± 0.07	2.05 ± 0.06	3.7 ± 0.4	M	(20)	1,810 ± 210			
						3.1 ± 0.4	SA	(6)	1,530 ± 220			
KERC5	Core	0.47 ± 0.02	0.50 ± 0.03	1.64 ± 0.08	1.89 ± 0.07	33 ± 3	SA	(6)	17,500 ± 1,800			
KERC4	Whole nest	0.47 ± 0.02	0.50 ± 0.03	1.64 ± 0.08	1.89 ± 0.07	45 ± 4	SA	(21)	23,800 ± 2,400			
						31 ± 3	SR	(15)	16,400 ± 1,800			

* Activities of ²³⁸U and ²³²Th decay series nuclides, and ⁴⁰K, are converted to dose rates (mean ± total uncertainty) using published relations^{24,25}.

† Estimate (1) is the sum of the *in situ* γ-ray dose rate (determined using a field γ-spectrometer, and high-resolution γ-spectrometry on powdered rock samples²⁶), and the cosmic-ray dose rate estimated from published equations²⁷. Estimate (2) is obtained from thermoluminescence dosimetry capsules (CaF₂ in copper) attached to the rock face for one year after nest collection (and corrected for travel dose, self-dose, and capsule-wall attenuation¹⁵).

‡ Mean palaeodose ± standard error, obtained by multiple-aliquot additive-dose (M), single-aliquot additive-dose (SA), and single-aliquot regenerative-dose (SR) protocols. Values in parentheses are the number of aliquots used for each multiple-aliquot determination of palaeodose, or the number of single-aliquot or single-grain (for KERC4) estimates of palaeodose.

§ Mean ± total uncertainty, calculated as the quadratic sum of the random and systematic uncertainties¹².

|| Activity as percentage modern carbon, measured using the tandem accelerator mass spectrometer at ANSTO (ref. 28). A δ¹³C value of −25‰ is assumed. Ages for samples OZC362, 363, 364, 358 and 360 are indistinguishable from modern, whereas uncalibrated ¹⁴C ages for OZC361, 357 and 359 are 1,310 ± 100, 990 ± 60 and 640 ± 95 years BP, respectively. We have not yet determined the cause(s) of the three non-modern ages, although finely comminuted contaminants^{29,30} are presumably involved.

distribution of single-grain palaeodoses is thereby obtained^{15,16}, enabling unilluminated grains to be discriminated from grains exposed to light near the surface.

The 'age profile' obtained for nest DR6 (Fig. 1, Table 1) indicates that only the outermost portion of the nest contains grains that have been exposed to modern sunlight, yielding an apparent age of 110 ± 20 years, with the next four portions having a weighted mean age of 270 ± 20 years. The two innermost portions have an average age of 610 ± 40 years, implying that DR6 consists of two generations of nest. This finding is consistent with the observation^{1,17} that mud-dauber wasps prefer to build nests near existing nests and on the stumps of abandoned nests, rather than on a bare rock surface. The non-zero age of the outermost portion corresponds to a residual palaeodose of 0.14 ± 0.02 Gy, and similar palaeodoses were obtained from the cores of the recent nest KERC9 and nest DR1. The ages of 100–150 years for KERC9, DR1 and DR2 indicate the lower age limit that can be expected using OSL. Two further lines of evidence support a recent origin for these nests: first, the presence of 160–270% ²¹⁰Pb excess (presumably from atmospheric fallout) in nests DR1 and KERC9 indicates that these nests are built from mud collected within the last 100 years; and second, AMS ¹⁴C determinations made on pollen grains⁸ (>5 µm in diameter) extracted from nests DR2 and KERC9 give ages that are indistinguishable from modern (Table 1), indicating that the pollen is derived from plants flowering around the time of nest construction.

The core of nest DR4 yielded an optical date of ~1,800 years which, although it is not obviously associated with any painting, demonstrates the preservation potential of large nests. This is further borne out by the remarkable antiquity of nests KERC4 and KERC5. The core of the latter yielded an age of ~17,500 years,

and the single grains dated from nest KERC4 suggest an age of more than 16,400 years for the underlying anthropomorphic painting. These results show that the mulberry-coloured human figure was painted at or before the Last Glacial Maximum, and that the underlying hand stencil must be older still. Independent support for a Pleistocene age is implied by the condition of equilibrium existing between ²³⁰Th and ²²⁶Ra in the mud from nest KERC5. Nests constructed in the past 600 years (DR1, DR2, DR6 and KERC9) exhibit disequilibrium between ²³⁰Th and ²²⁶Ra, owing to a 40–110% ²²⁶Ra excess associated with the sediments used to build the nests. If KERC5 and KERC4 had been constructed of mud with a similar initial ²²⁶Ra excess, it would have taken at least 8,000 years (five half-lives of ²²⁶Ra) for this excess to decay away.

Nests of *S. laetum* (including DR1, DR2, KERC5 and KERC9), *S. formosum* and eumenine wasps have been examined for pollen, spores and phytoliths. Pollen is present in nest mud chiefly as a result of wasps visiting flowering plants for nectar. Phytoliths provide a record of polymerized biogenic silica which has been deposited in the source mud after release from plant organic material. Initial investigation of a *S. laetum* nest and eumenine wasp nest (both undated but probably late Holocene in age) showed that both were rich in pollen, yielding 1–2 mg of pollen from 1 g of mud. A large, distinctive pollen type produced by *Grevillea* or *Hakea* was predominant in the *S. laetum* nest, whereas a more equal representation of pollen types (eucalypt, ti-tree, paperbark, wattle, *Grevillea* and *Hakea*) was present in the eumenine wasp nest (Table 2). A diverse mix of abundant pollen was found subsequently in nests DR1, DR2 and KERC9, but an indurated *S. formosum* nest seemed to contain no pollen. Pleistocene nest KERC5 showed the presence of some Myrtaceae pollen, but not enough to allow AMS ¹⁴C dating. These differences almost certainly reflect the particular foraging strategies of mud-dauber and potter wasps and perhaps some degradation of pollen with time. The types of phytolith extracted¹⁸ from the mud of four dated *S. laetum* nests (Table 3) include varieties of grass and dicotyledon, as well as carbon particles, starch grains and, at the Wandjina site, freshwater sponge spicules. These samples did not contain a sufficient mass of phytolith for AMS ¹⁴C dating, but other nests may prove amenable. Previous studies have used occluded carbon in phytoliths for AMS ¹⁴C dating and palaeoclimatic reconstructions¹⁹. Further investigations of fossil pollen and phytolith types should allow a

Table 2 Pollen and spore types extracted from selected mud-wasp nests

Taxon	Main pollinator	Total pollen and spore count (%)		
		Eumenine wasp nest*	Sceliphron laetum nest*	Nest KERC5
<i>Eucalyptus</i> type	insect	24	2	28
<i>Leptospermum</i> type	insect	15	1	–
<i>Melaleuca</i> type	insect	10	<1	–
Other Myrtaceae	insect	5	<1	51
<i>Acacia</i>	insect	38	1	2†
<i>Grevillea/Hakea</i>	insect	4	91	2†
Apiaceae	insect	1	<1	–
Chenopodiaceae	wind	–	<1	–
Asteraceae	insect	<1	<1	–
Haloragaceae	insect	<1	1	–
Poaceae	wind	–	2	2
Other angiosperms	insect	3	1	15†
Ferns	water	<1	<1	–
<i>n</i> §		2,357	1,875	47

Types not recorded are indicated by a dash.

* Nests are probably late Holocene in age.

† Modern contaminants, mostly in the 25–45 µm fraction.

§ *n*, Number of pollen grains and spores counted.

Table 3 Phytolith types extracted from selected *S. laetum* nests

Phytolith type	Nest DR1	Nest DR2	Nest KERC9	Nest KERC5
Grasses*				
B	–	Y	Y	Y
A	–	–	–	Y
AT	–	–	Y	–
OR	–	Y	Y	–
Dicotyledon†				
R	Y	Y	Y	Y
O	Y	–	Y	Y
Carbon particles	Y	Y	Y	–
Starch grains‡	20	20	20	16
Sponge spicules	Y	Y	–	–

Types recorded are indicated Y; types not recorded are indicated by a dash.

* Grass shapes: B, bilobe; A, angular; AT, arc/triangle; OR, ornamented rectangles.

† Dicotyledon and non-Poaceae monocotyledon shapes: R, rectangular; O, others.

‡ Approximate grain diameter (µm).

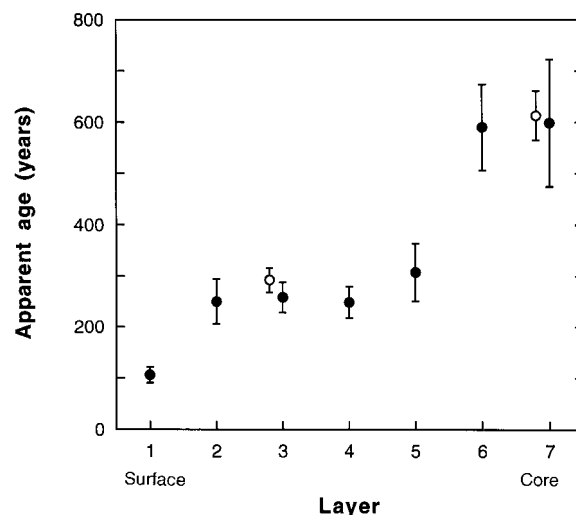


Figure 1 Optical dates obtained from each portion of *S. laetum* nest DR6 (40 mm from core to surface). Filled circles, mean ages ($\pm 1\sigma$ uncertainties) derived using a multiple-aliquot additive-dose protocol; open circles, mean ages derived using a single-aliquot additive-dose protocol. Note the concordance between multiple-aliquot and single-aliquot age estimates for layers 3 and 7.

detailed reconstruction of the palaeoenvironment for each interval of time represented by a nest.

The worldwide distribution of mud-wasps and the demonstrated longevity of their nests should prove a valuable tool in archaeological and palaeoclimatic research. The rapid formation and short period of usage of a nest, and the incorporation of a variety of palaeoecological indicators, makes each mud-wasp nest a 'snapshot' of its late Quaternary environment. By examining nests of different ages in several localities, it should be feasible to infer sequences of prehistoric human activity and vegetation change at various geographic scales and in climatic zones where other palaeoecological records are poorly preserved. Optical dating of single grains of sand now makes it possible to date very small nests, such as those produced by *S. formosum* (which typically overlie the stylistically oldest paintings), and the painted stumps of nests (to obtain maximum ages for art motifs). Sediment accretions created by some other animals (such as ants, termites and mud-nesting birds) may also be suitable for optical dating if the mineral grains were fully exposed to sunlight during final emplacement. □

Methods

Palaeodose determinations. Quartz grains 90–125 µm in diameter were extracted from the mud nests and then etched in 40% hydrofluoric acid for 45 min to remove the α -particle-dosed outer rinds. Multiple-aliquot additive-dose palaeodoses were obtained using conventional quartz dating procedures^{6,20,21}, a 300-s preheat at 220 °C, optical stimulation (500 ± 40 nm) at room temperature, and OSL emissions detected through 2.5-mm U-340 and 3-mm UG-11 filters. Single-aliquot additive-dose palaeodoses were obtained from successive short-shine/dose/preheat cycles, using a 10-s preheat at 160–300 °C (the preheat plateau⁶ region was determined for each multi-grain sample and invariably included 280 °C), optical stimulation of 420–550 nm at 125 °C, and OSL emissions detected through two 3-mm U-340 filters^{14,15,21}. A 10-s preheat at 280 °C was used for all single-grain analyses. Single-aliquot regenerative-dose palaeodoses were obtained using the same equipment, the total OSL signal from successive long-shine/dose/preheat cycles, and the 110 °C thermoluminescence signal to correct for differences in OSL sensitivity between cycles¹⁵. Laboratory ⁹⁰Sr/⁹⁰Y sources delivered β -particle doses at 22–48 mGy s⁻¹. OSL ages for nest KERC4 are calculated from individual grains with palaeodoses >10 Gy. The additive-dose and regenerative-dose palaeodoses (from 21 and 15 grains, respectively) show a broad, approximately gaussian, frequency distribution, which we interpret as reflecting grain-to-grain differences in dose-response behaviour and β -particle microdosimetry¹⁵. Grains with palaeodoses >10 Gy (33 grains with palaeodoses of 14–59 Gy and three grains with additive-dose palaeodoses of ~75 Gy) are presumed to be light-safe, whereas those with a smaller palaeodose (25% of the grains examined) are considered to have been exposed to modern sunlight, either on the surface of the nest or in the immediate subsurface where attenuated sunlight may penetrate sufficiently to partly empty the OSL traps. This problem does not apply to nest KERC5, from which grains were extracted only from the unilluminated core.

Dose rate determinations. The γ -ray dose is derived mostly from the local bedrock, with a minor (5%) γ -ray contribution from the nest mud. The cosmic-ray plus bedrock γ -ray dose rate was estimated by two independent methods for most nests (Table 1 footnote), and averaged to calculate nest ages. The β -particle dose rate is derived entirely from the nest mud and was calculated using an attenuation factor²² of 0.93 ± 0.03 . The only exceptions are layer 1 of DR6 (grains in this thin exterior layer are assumed to have received only 90% of the 4π β -particle dose rate), and nests KERC5 and KERC4 (for which a 25% contribution from bedrock (β -particle dose rate, 0.49 ± 0.03 mGy yr⁻¹) is assumed, owing to the close contact between the sampled grains and the rock surface, giving a total β -particle dose rate 18% smaller than if the β -particle flux were derived from the mud only). The β -particle dose rates from nest mud were deduced from X-ray fluorescence and α -particle spectrometry²³. The latter analyses show that the ²³⁸U decay series is in disequilibrium, with unsupported excesses of ²¹⁰Pb (in DR1 and KERC9) and ²²⁶Ra (in DR1, DR2, DR6 and KERC9). A ²¹⁰Pb excess does not significantly affect the dose rate, and had KERC5 or KERC4 been formed with an initial ²²⁶Ra

excess of 100% then the dose rate (integrated over 20,000 years) would be <1% greater than the modern (equilibrium) dose rate used to calculate their OSL ages. The ²¹⁰Pb/²²⁶Ra ratio for DR6 indicates ²²²Rn emanation of 18%, which is assumed to have prevailed since nest construction. Because of the paucity of mud available for KERC4, the nest-derived β -particle dose rate is assumed to be the same as that determined for its lateral extension, KERC5. An internal α -particle and absorbed β -particle dose rate²² of 0.02 ± 0.01 mGy yr⁻¹ was deduced from U and Th concentrations in single grains of acid-etched quartz (measured by laser-ablation inductively coupled plasma mass spectrometry (ICP-MS) using a pulsed ArF excimer laser operating at 193 nm) and an assumed α -particle efficiency 'a' value of 0.1. The dose rates require no moisture corrections as the nests are dry.

Received 4 February; accepted 17 April 1997.

1. Naumann, I. D. in *The Rock Art Sites of Kakadu National Park – Some Preliminary Research Findings for their Conservation and Management* (ed. Gillespie, D.) 127–189 (Special Publication 10, Australian National Parks and Wildlife Service, Canberra, 1983).
2. Gauld, I. D. & Bolton, B. (ed.) *The Hymenoptera* (Oxford Univ. Press, 1988).
3. LaSalle, J. & Gauld, I. D. (ed.) *Hymenoptera and Biodiversity* (C.A.B. International, Wallingford, 1993).
4. Naumann, I. D. in *The Insects of Australia: A Textbook for Students and Research Workers* 2nd edn, 916–1,000 (Melbourne Univ. Press, 1991).
5. Huntley, D. J., Godfrey-Smith, D. I. & Thewalt, M. L. W. Optical dating of sediments. *Nature* **313**, 105–107 (1985).
6. Aitken, M. J. Optical dating: a non-specialist review. *Quat. Sci. Rev.* **13**, 503–508 (1994).
7. Duller, G. A. T. Recent developments in luminescence dating of Quaternary sediments. *Prog. Phys. Geogr.* **20**, 127–145 (1996).
8. Brown, T. A., Nelson, D. E., Mathewes, R. W., Vogel, J. S. & Southon, J. R. Radiocarbon dating of pollen by accelerator mass spectrometry. *Quat. Res.* **32**, 205–212 (1989).
9. Walsh, G. L. *Bradshaws: Ancient Rock Paintings of North-West Australia* (The Bradshaw Foundation, Geneva, 1994).
10. Morwood, M. J., Walsh, G. L. & Watchman, A. The dating potential of rock art in the Kimberley, N.W. Australia. *Rock Art Res.* **11**, 79–87 (1994).
11. Smith, F. in *Catalogue of Hymenopterous Insects in the Collection of the British Museum Part IV* 207–497 (British Museum, London, 1856).
12. Aitken, M. J. *Thermoluminescence Dating* (Academic, London, 1985).
13. Duller, G. A. T. Luminescence dating using single aliquots: methods and applications. *Radiat. Meas.* **24**, 217–226 (1995).
14. Murray, A. S., Roberts, R. G. & Wintle, A. G. Equivalent dose measurement using a single aliquot of quartz. *Radiat. Meas.* **27**, 171–184 (1997).
15. Murray, A. S. & Roberts, R. G. Determining the burial time of single grains of quartz using optically stimulated luminescence. *Earth Planet. Sci. Lett.* (submitted).
16. Lamothe, M., Balescu, S. & Auclair, M. Natural IRSI intensities and apparent luminescence ages of single feldspar grains extracted from partially bleached sediments. *Radiat. Meas.* **23**, 555–561 (1994).
17. Waterhouse, D. F. in *The Insects of Australia: A Textbook for Students and Research Workers* 2nd edn, 221–235 (Melbourne Univ. Press, 1991).
18. Bowdery, D. E. thesis, Australian National Univ. (1996).
19. Kelly, E. F., Amundson, R. G., Marino, B. D. & Deniro, M. J. Stable isotope ratios of carbon in phytoliths as a quantitative method of monitoring vegetation and climate change. *Quat. Res.* **35**, 222–233 (1991).
20. Roberts, R. G. *et al.* The human colonisation of Australia: optical dates of 53,000 and 60,000 years bracket human arrival at Deaf Adder Gorge, Northern Territory. *Quat. Sci. Rev.* **13**, 575–583 (1994).
21. David, B., Roberts, R., Tuniz, C., Jones, R. & Head, J. New optical and radiocarbon dates from Ngarrabullgan Cave, a Pleistocene archaeological site in Australia: implications for the comparability of time clocks and for the human colonization of Australia. *Antiquity* **71**, 183–188 (1997).
22. Mejdahl, V. Thermoluminescence dating: beta-dose attenuation in quartz grains. *Archaeometry* **21**, 61–72 (1979).
23. Martin, P. & Hancock, G. Routine analysis of naturally occurring radionuclides in environmental samples by alpha-particle spectrometry. Supervising Scientist for the Alligator Rivers Region, Research Report 7 (Australian Government Publishing Service, Canberra, 1992).
24. Nambi, K. S. V. & Aitken, M. J. Annual dose conversion factors for TL and ESR dating. *Archaeometry* **28**, 202–205 (1986).
25. Olley, J. M., Murray, A. & Roberts, R. G. The effects of disequilibrium in the uranium and thorium decay chains on burial dose rates in fluvial sediments. *Quat. Sci. Rev.* **15**, 751–760 (1996).
26. Murray, A. S., Marten, R., Johnston, A. & Martin, P. Analysis for naturally occurring radionuclides at environmental concentrations by gamma spectrometry. *J. Radioanal. Nuclear Chem. Articles* **115**, 263–288 (1987).
27. Prescott, J. R. & Hutton, J. T. Cosmic ray contributions to dose rates for luminescence and ESR dating: large depths and long-term time variations. *Radiat. Meas.* **23**, 497–500 (1994).
28. Tuniz, C. *et al.* The ANTARES AMS Centre: a status report. *Radiocarbon* **37**, 663–673 (1995).
29. Brown, T. A., Farwell, G. W., Grootes, P. M. & Schmidt, F. H. Radiocarbon AMS dating of pollen extracted from peat samples. *Radiocarbon* **34**, 550–556 (1992).
30. Long, A., Davis, O. K. & De Lanois, J. Separation and ¹⁴C dating of pure pollen from lake sediments: nanofossil AMS dating. *Radiocarbon* **34**, 557–560 (1992).

Acknowledgements. We thank the traditional Aboriginal custodians (M. Pandilow, T. H. Unghango and family members, L. Utemara and D. Utemara, L. Karedada, J. Karedada and S. Mangolamara), Kalumburu Community Council and A. Koeys for permission to undertake this study. Samples were collected under permits 166 and NE001023 from the Western Australian Aboriginal Affairs Department and Department of Conservation and Land Management, respectively. We thank D. Questiaux for preparing and E. Haskell for analysing the thermoluminescence dosimetry capsules; M. Shelley for preparing and L. Kinsley for measuring the laser-ablation ICP-MS samples; K. Weiss and G. Atkin for pollen extraction; G. Jacobsen for ¹⁴C sample preparation; M. J. Olley for X-ray fluorescence analyses; and A. Bell and M. Aitken for comments. R.R. acknowledges the support of a Queen Elizabeth II fellowship from the Australian Research Council and a J. G. Russell Award from the Australian Academy of Science, which funded some of the research costs. The Bradshaw Foundation, Australian National University and Australian Research Council funded the fieldwork, and the Australian Institute of Nuclear Science and Engineering funded the AMS ¹⁴C determinations.

Correspondence and requests for materials should be addressed to R.R. (e-mail: bert.roberts@latrobe.edu.au).

Role of mutator alleles in adaptive evolution

F. Taddei^{*#}, M. Radman^{*}, J. Maynard-Smith[†],
B. Toupance[‡], P. H. Gouyon[‡] & B. Godelle^{‡§}

^{*} Laboratoire de Mutagenèse, Institut J. Monod, CNRS Université Paris 7, 2 Place Jussieu, 75251 Paris, France

[†] University of Sussex, Microbial Genetics Group, School of Biological Sciences, Falmer, Brighton BN1 9QG, UK

[‡] Université de Paris-Sud CNRS, Laboratoire Évolution et Systématique, Bat. 362 F91405 Orsay cedex, France

[§] Institut National Agronomique Paris-Grignon, 16 rue Claude Bernard, 75005 Paris and # Ecole Nationale du Génie Rural, des Eaux et des Forêts, 19 avenue du Maine, 75732 Paris, France

Because most newly arising mutations are neutral or deleterious, it has been argued¹⁻³ that the mutation rate has evolved to be as low as possible, limited only by the cost of error-avoidance and error-correction mechanisms. But up to one per cent of natural bacterial isolates are 'mutator' clones that have high mutation rates⁴⁻⁶. We consider here whether high mutation rates might play an important role in adaptive evolution. Models of large, asexual, clonal populations adapting to a new environment show that strong mutator genes (such as those that increase mutation rates by 1,000-fold) can accelerate adaptation, even if the mutator gene remains at a very low frequency (for example, 10^{-5}). Less potent mutators (10 to 100-fold increase) can become fixed in a fraction of finite populations. The parameters of the model have been set to values typical for *Escherichia coli* cultures, which behave in a manner similar to the model in long-term adaptation experiments⁷.

Early models of the evolution of the mutation rate were based on group selection for an optimal compromise between adaptability and adaptedness^{2,3}. However, later models, incorporating mutators and antimutators (modifiers of the mutation rate) showed that a mutator can reduce individual fitness while increasing the probability for an adaptive mutation to appear in the population. The prediction of these models was that a minimal mutation rate would be selected in a stable environment (reduction principle⁸), whereas in an oscillating environment, infinite populations at equilibrium could have a non-minimal mutation rate^{3,9,10}.

However, most of evolution may take place with the population being far from equilibrium, with organisms facing an ever-changing

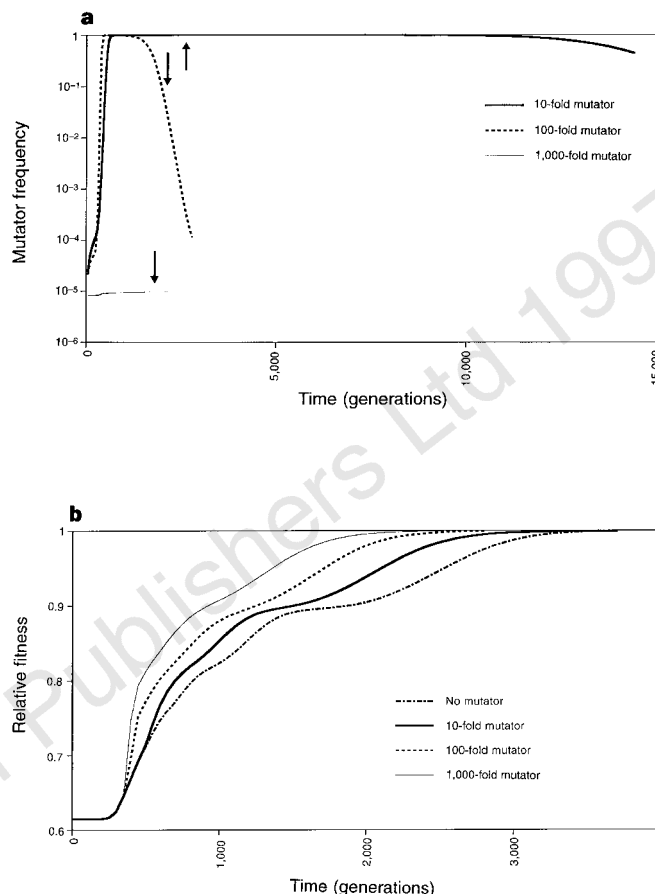


Figure 2 Mutators speed up evolution even when they are not fixed in infinite populations. Major effect mutators remain at very low frequency (**a**) but allow rapid fitness increase of the whole population (**b**) thanks to reversion to non-mutator genotypes associated with favourable mutations. **a**, Evolution of the frequency of mutator genotypes. Intermediate strength mutators become fixed, and then disappear from the population when all favourable mutations are fixed (an arrow symbolizes the time when relative fitness has reached 0.99, generation 3,100, 2,650, 2,200 and 1,850 for populations with no mutator, a 10-, a 100- and a 1,000-fold mutator, respectively.) **b**, Evolution of the mean fitness of asexual populations subjected to mutation and selection.

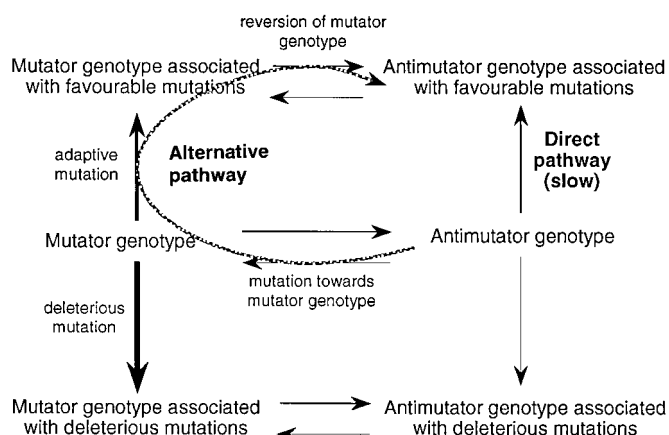


Figure 1 Mutators as a fast track to generate adaptive mutations. Fixation of adaptive mutations can proceed in two ways: favourable mutants appear in a non-mutator genetic background, and then spread by selection; mutator genotypes, obtained by mutating an antimutator gene (such as a mismatch repair gene) can also be transiently involved by generating favourable mutations and then reverting to the wild-type allele. (See Methods for values of parameters.)

environment¹¹, a situation that favours a non-minimal mutation rate^{12–14}. Previous studies did not consider the effects of random drift. In fact, finite population size with moderate mutation rates maintains favourable mutants at such low frequencies that they are absent in many natural populations. Experiments with *Escherichia coli* held for many generations in a chemostat lead to a paradoxical unpredicted outcome: a mutator allele (monitored by the use of a linked selectable marker) increased in frequency when abundant but decreased in frequency when rare^{15–19}. The present work targets the difference between finite and infinite populations under directional selection. Our model uses mutation rates typical for *E. coli* (see Fig. 1 for a general overview and Methods for details), using the large amount of mutagenesis data available for this organism (see refs 1, 20).

In the absence of selection for adaptive mutations, a mutation/selection equilibrium is reached where the spontaneous generation of mutator alleles by inactivation of an 'antimutator' gene at a frequency of 5×10^{-7} is balanced by increased production of deleterious and lethal mutations by the mutator (the cost of

deleterious mutations was set at 0.05). The equilibrium value observed in the simulations was, respectively, 6×10^{-4} , 6×10^{-5} and 10^{-5} for 10-, 100- and 1,000-fold mutator alleles.

The evolution of a population can be very different when adapting to a new environment if adaptation is limited by mutation availability. Mutator genotypes generate adaptive mutations efficiently. Therefore we suggest that a transient increase in mutator frequencies should be a common event in natural asexual populations subjected to sporadic adaptive evolution (Fig. 1).

We modelled this by assuming that favourable alleles were possible in 15 to 39 loci, with selective advantages ranging from 0.005 to 0.03, values that are consistent with those observed in long-term experiments with *E. coli*^{21,22}. In an infinite population, 10- and 100-fold mutator alleles can be fixed by 'hitch-hiking' with favourable mutations in about 500 generations (Fig. 2a), whereas a 1,000-fold mutator will not be fixed during the course of evolution.

However, provided there are enough adaptive mutations, there is a positive correlation between the speed of increase in fitness and the strength of the mutator allele. A population containing a 1,000-fold

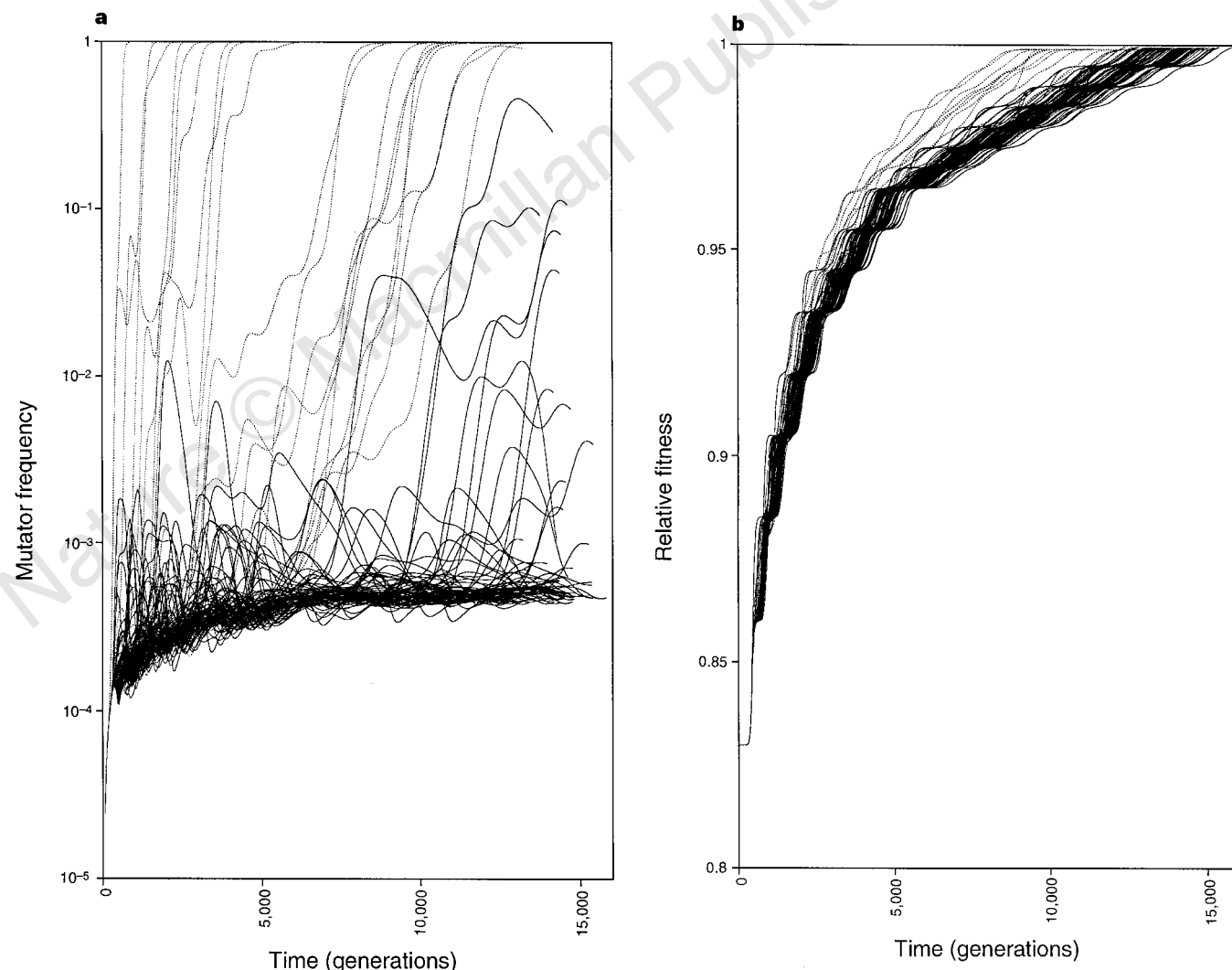


Figure 3 Intermediate mutators can be fixed during adaptation of finite size populations (10-fold mutators are shown). The model is similar to the one described in Fig. 2. Here, however, finite populations were allowed to grow exponentially from 10^3 to 10^{10} individuals and then were diluted back to 10^8 , with random sampling occurring every 7 generations for frequency classes lower than 13×10^{-8} (see Methods for more details about the sampling procedure). Each of

100 independent simulations is represented on these plots. Dotted lines symbolize the populations where the mutator allele has reached over 50%. **a**, Change in mutator frequency. Mutators can be favoured by hitch-hiking with adaptive mutations and thus become fixed in the population. **b**, Evolution of the mean fitness of populations subjected to mutation, selection and drift. Fixation of mutator genotypes can accelerate fitness increase.

mutator evolves much faster than a population without a mutator (Fig. 2b). The fact that an allele can have such a dramatic effect, and yet remain rare, can be explained by its high reversion rate (we assumed a point mutation that can easily be reverted by such a strong mutator), which eliminates linkage disequilibrium with favourable mutations. When its reversion rate was set to zero (modelling a deletion of the antimutator gene), the presence of a strong mutator at low frequency did not improve the rate of increase of the fitness of the population, because the favourable mutants produced were handicapped by continued linkage to this mutator allele and thus by its deleterious effects on the whole genome. Once the adaptive peak has been reached, the frequency of the mutator will decline (Fig. 2a), albeit very slowly for a 10-fold mutator, which requires about 20,000 generations to reach its equilibrium value, thus showing a strong hysteresis effect.

After modelling infinite populations, we have simulated more realistic finite populations. In the colon of mammals, population sizes of *E. coli* vary between 10^6 and 10^8 cells per g of intestinal content²³, whereas in a liquid culture density typically reaches 10^9 ml⁻¹. Finite size populations were simulated by periodical probabilistic samplings (see details below). Repeated simulations of a finite population, oscillating between 10^8 and 10^{10} cells, showed stochastic behaviour (Fig. 3a). Mutators with 10- and 100-fold effects reached a frequency of above 50% in a fraction of the trials (19 and 7% respectively). On average the fitness of populations with a high frequency of mutators increased faster during the process of adaptation (Fig. 3b). However, once all adaptive mutations were fixed, the load of deleterious mutations generated by the mutator, that had not yet reverted, lowered the fitness of the populations (Fig. 3a).

The stochastic pattern observed in Fig. 3a is due to the low probability that a mutator present in a small population will produce a favourable mutation. Once this happens, the mutator allele increases in frequency by hitch-hiking, thus facilitating the appearance of a second adaptive mutation. Mutators can then become fixed by this reiterated process, which is hampered by reversion to the non-mutator genotype. These patterns, obtained as a result of drift in finite populations, can explain why mutators have a high probability of disappearing when rare, but increase in frequency when abundant.

One of the main assumptions that we have made in this analysis is the absence of recombination, which would disrupt the hitch-hiking effect of the mutator and the linkage of favourable with deleterious mutations. Some bacterial populations are largely asexual, and these results should apply to them, but for bacteria with high levels of genetic exchange²⁴ further modelling is needed; such a model should take into account that some mutator alleles increase the rate of these genetic exchanges (see ref. 25 for a review) and that sex might allow the recovery of favourable mutations from a deleterious background²⁶. The high frequency of mutators associated with pathogenic bacteria⁶ suggests that the evolution of increased mutation rates is relevant to the evolution of parasites¹³. Our conclusions might also apply to clonal eukaryotic lineages such as large populations of autogamous plants (similarly, the outcrossing rate of autogamous plants can increase under directional selection²⁷) or tumours where mutators are associated with malignancy²⁸, but key parameters are not as well known as in the case of bacteria, and accurate modelling will have to be delayed.

Furthermore, these results show that during the course of evolution, where phases of adaptation and stasis alternate²⁹, there is no pure strategy of mutation rate. Rather, selection seems to result in an alternation of high and low mutation rate through forward and reverse mutations at the mutator locus. Alternatively, transient mutators²⁰ might allow clonal populations to enhance their adaptability by increasing their mutation rate: for example, inducible mechanisms (see ref. 30 for a review) might be particularly useful in response to stress. □

Methods

Mutation rates and selection. Mutation occurs at fixed rates (deleterious mutations, 10^{-4} ; lethal mutations, 10^{-5} ; favourable mutations, 10^{-8}). All mutation rates are increased by a given factor m in the mutator genotype. Exchange between the mutator and the wild type occurs by mutation at fixed rates (5×10^{-7} forward and $5 \times 10^{-10} \times m$ reverse). Each deleterious mutation causes an additive loss of fitness of 0.05. Different adaptive mutations are possible, allowing an additive increase in fitness of 0.005, 0.01, 0.015, 0.02, 0.025, 0.03, respectively. In infinite populations there were 21, 8, 4, 3, 2 and 1, respectively, of each class whereas in finite populations these numbers were limited to 7, 3, 2, 1, 1 and 1.

Sampling procedure used to model drift. After each 7 generations, we used a Poisson sampling procedure except for classes larger than 13 individuals, for which the size of the sample was set to the expected value (for these classes, the probability that a genotype is lost by drift is lower than 10^{-6}).

Received 18 December 1996; accepted 20 March 1997.

- Drake, J. W. A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl Acad. Sci. USA* **88**, 7160–7164 (1991).
- Kimura, M. On the evolutionary adjustment of spontaneous mutation rates. *Genet. Res.* **9**, 23–34 (1967).
- Leigh, E. G. The evolution of mutation rates. *Genetics* **73**, 1–18 (1973).
- Jysum, K. Observation of two types of genetic instability in *Escherichia coli*. *Acta Pathol. Microbiol. Immunol. Scand.* **48**, 113–120 (1960).
- Gross, M. D. & Siegel, E. C. Incidence of mutator strains in *Escherichia coli* and coliforms in nature. *Mutat. Res.* **91**, 107–110 (1981).
- LeClerc, J. E., Li, B., Payne, W. L. & Cebula, T. A. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. *Science* **274**, 1208–1211 (1996).
- Snigowski, P. D., Gerrish, P. J. & Lenski, R. E. Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* **387**, 703–705 (1997).
- Liberman, U. & Feldman, M. W. Modifiers of mutation rate: a general reduction principle. *Theor. Pop. Biol.* **30**, 125–142 (1986).
- Leigh, E. G. Natural selection and mutability. *Am. Nat.* **104**, 301–305 (1970).
- Ishii, K., Matsuda, H., Iwasa, Y. & Sasaki, A. Evolutionary stable mutation rate in a periodically changing environment. *Genetics* **121**, 163–174 (1989).
- Van Valen, L. A new evolutionary law. *Evol. Theory* **1**, 1–30 (1973).
- Moxon, E. R., Rainey, P. B., Nowak, M. A. & Lenski, R. E. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr. Biol.* **4**, 24–33 (1994).
- Harauchi, Y. & Sasaki, A. Host-parasite arms race in mutation modifications: indefinite escalation despite a heavy load. *J. Theor. Biol.* **183**, 121–137 (1996).
- Mao, E. F., Lane, L., Lee, J. & Miller, J. H. Proliferation of mutators in a cell population. *J. Bacteriol.* **179**, 417–422 (1997).
- Chao, L. & Cox, E. C. Competition between high and low mutating strains of *Escherichia coli*. *Evolution* **37**, 125–134 (1983).
- Tröbner, W. & Piechocki, R. Competition growth between *Escherichia coli* *mutL* and *mut+* in continuously growing cultures. *Z. Allg. Mikrobiol.* **21**, 347–349 (1981).
- Tröbner, W. & Piechocki, R. Competition between isogenic *mutS* and *mut+* populations of *Escherichia coli* K12 in continuously growing cultures. *Mol. Gen. Genet.* **198**, 175–176 (1984).
- Tröbner, W. & Piechocki, R. Selective advantage of *polA1* mutator over *polA+* strains of *Escherichia coli* in a chemostat. *Naturwissenschaften* **72**, 377–378 (1985).
- Chao, L., Vargas, C., Spear, B. B. & Cox, E. C. Transposable elements as mutator genes in evolution. *Nature* **303**, 633–635 (1983).
- Ninio, J. Transient mutators: a semiquantitative analysis of the influence of translation and transcription errors on mutation rates. *Genetics* **129**, 957–962 (1991).
- Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. Long-term experimental evolution in *E. coli*. I. Adaptation and divergence during 2000 generations. *Am. Nat.* **138**, 1315–1341 (1991).
- Lenski, R. E. & Travisano, M. Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc. Natl Acad. Sci. USA* **91**, 6808–6814 (1994).
- Hartl, D. L. & Dykhuizen, D. E. The population genetics of *Escherichia coli*. *Annu. Rev. Genet.* **18**, 31–68 (1984).
- Maynard-Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. How clonal are bacteria? *Proc. Natl Acad. Sci. USA* **90**, 4384–4388 (1993).
- Matic, I., Taddei, F. & Radman, M. Genetic barriers among bacteria. *Trends Microbiol.* **4**, 69–73 (1996).
- Peck, J. R. A ruby in the rubbish: beneficial mutations, deleterious mutations and the evolution of sex. *Genetics* **137**, 597–606 (1994).
- David, J. L., Savy, Y. & Brabant, P. Outcrossing and selfing evolution in populations under directional selection. *Heredity* **71**, 642–651 (1993).
- Tomlinson, I. P. M., Novelli, M. R. & Bodmer, W. F. The mutation rate and cancer. *Proc. Natl Acad. Sci. USA* **93**, 14800–14803 (1996).
- Magnasco, M. O. & Thaler, D. S. Changing the pace of evolution. *Phys. Lett. A* **221**, 287–292 (1996).
- Taddei, F., Vulic, M., Radman, M. & Matic, I. In *Environmental Stress, Adaptation and Evolution* (eds Bijlsma, K. & Loeschcke, V.) (Birkhäuser, Basel, in the press).

Acknowledgements. Most of the simulations used the SP2 computer, at the Centre de Ressources Informatiques de l'Université de Paris-Sud (Orsay). We are grateful to M.-P. Donsimoni for providing us with a favourable environment and to J. Shykoff and N. Smith for eliminating deleterious style and generating favourable comments. This work has been funded by 'Bureau des Ressources Génétiques, Ministère de l'Environnement, Association de la Recherche contre le Cancer, Actions Concertées Coordonnées-Sciences du Vivant du Ministère de l'Enseignement Supérieur et de la Recherche and Groupement de Recherche et d'Études sur les Génomes'.

Correspondence should be addressed to F.T. (e-mail: taddei@ijm.jussieu.fr). Request for the C code used to model mutators should be sent to B.G. (e-mail: godelle@psisun.u-psud.fr).

Evolution of high mutation rates in experimental populations of *E. coli*

Paul D. Sniegowski*, Philip J. Gerrish† & Richard E. Lenski†

* Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

† Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824, USA

Most mutations are likely to be deleterious, and so the spontaneous mutation rate is generally held at a very low value¹. Nonetheless, evolutionary theory predicts that high mutation rates can evolve under certain circumstances^{2–4}. Empirical observations have previously been limited to short-term studies of the fates of mutator strains deliberately introduced into laboratory populations of *Escherichia coli*^{5–7}, and to the effects of intense selective events on mutator frequencies in *E. coli*⁸. Here we report the rise of spontaneously originated mutators in populations of *E. coli* undergoing long-term adaptation to a new environment. Our results corroborate computer simulations of mutator evolution in adapting clonal populations⁴, and may help to explain observations that associate high mutation rates with emerging pathogens⁹ and with certain cancers¹⁰.

As a means of studying evolution directly in the laboratory, 12 replicate experimental populations of a clonal strain of *E. coli* were established¹¹. Because these populations were founded from a single ancestral clone, mutation provided their only source of genetic variation. The glucose-limited environment in which these populations were propagated was essentially novel at the outset of the experiment and it provided considerable scope for adaptive evolution. Substantial evolutionary increases in fitness and changes in certain other phenotypic features in these populations have been documented elsewhere^{11–15}.

We measured mutation rates in the common ancestral strain and in the 12 experimental populations after they had been evolving for 10,000 generations. Most of the populations retained the ancestral mutation rate, but three populations (designated Ara[–]2, Ara[–]4 and Ara[–]6) displayed mutation rates that were between one and two orders of magnitude higher than those in the ancestor (Fig. 1). The times at which mutator phenotypes arose during the evolution of populations Ara[–]2, Ara[–]4 and Ara[–]6, as determined by screening for the mutator phenotype in isolates stored periodically throughout the experiment, are shown in Fig. 2. Once a lineage displayed a mutator phenotype, all subsequent isolates from that lineage also tested as mutators through 10,000 generations. This observation indicates that mutators rose to and remained at high frequencies in these populations.

To ascertain a genetic basis for the observed mutator phenotypes, we transformed 10,000-generation clonal isolates from populations Ara[–]2, Ara[–]4 and Ara[–]6, and an isolate from the ancestral strain REL606 with multicopy plasmids bearing wild-type alleles of seven known general mutator loci. The ancestral mutation rate was fully restored in the Ara[–]2, Ara[–]4 and Ara[–]6 strains only by the presence of wild-type alleles of genes in the methyl-directed mismatch repair pathway¹⁶ (Fig. 3). The mutation rate in the ancestral strain, REL606, was not significantly affected by the plasmids (with a single exception: see Fig. 3). In the Ara[–]6 strain the *mutS*⁺ allele alone restored the ancestral mutation rate. In the Ara[–]2 and Ara[–]4 strains the ancestral rate was restored completely by *uvrD*⁺, and the data also indicated a partial effect of *mutL*⁺. Recent studies have suggested that there is a mechanistic interaction between the *mutL*

and *uvrD* gene products, such that a defect in one may in some cases be complemented by increased production of the other (P. Modrich, personal communication).

Most mutations are deleterious, so mutator alleles are likely on average to have negative effects on fitness. In an evolving clonal population, however, a deleterious mutator can rise to high frequency (hitch-hike) in association with an adaptive mutation, provided that the selective cost of the mutator does not outweigh the selective benefit of the adaptive mutation. Hitch-hiking of mutators with adaptive mutations was demonstrated previously in chemostat populations of *E. coli*⁶, but these results left open the question of whether such events would be likely to occur in natural populations. When a mutator was introduced above a relatively high threshold number relative to the wild type, the mutator population always acquired an adaptive mutation before the wild-type population, and the mutator rose to high frequency. However, when the mutator was introduced in lower numbers, the wild-type population always acquired a beneficial mutation first and displaced the mutator.

In the evolution experiment we have described here, as in natural populations, mutator alleles must have arisen by mutation. This raises the question of how mutators reached high frequencies in 3 of our 12 populations. Computer simulations⁴ and analytical modelling (P.J.G. and P.D.S., unpublished data) suggest that rare mutators may occasionally hitch-hike to high frequencies in finite asexual populations as a consequence of chance associations with adaptive mutations. We think that such chance hitch-hiking events are the likely explanation for our results. Sufficient time may not have elapsed for them to have been observed in earlier studies of

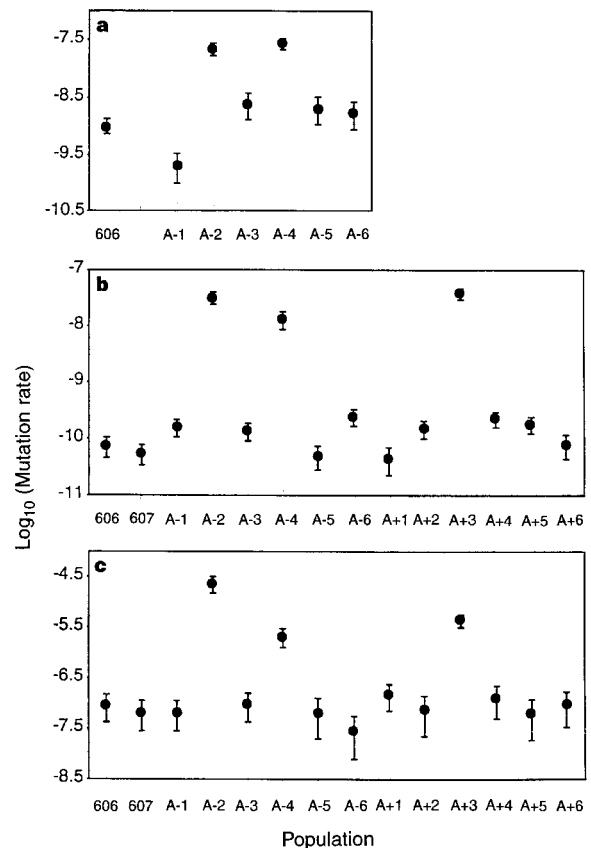


Figure 1 Rates of mutation in 12 Ara[–] and Ara⁺ experimental populations (A – 1 to A – 6 and A + 1 to A + 6) at 10,000 generations and in their common ancestors REL606 (Ara[–]) and REL607 (Ara⁺). Error bars give approximate 95% confidence intervals. **a**, Reversion to Ara⁺ in isolates from the 7 Ara[–] populations. **b**, Mutation to nalidixic-acid resistance. **c**, Mutation to bacteriophage T5 resistance.

competition between mutator and wild-type strains⁵⁻⁷, as those studies were carried out for only a few tens or hundreds of generations, as opposed to the 10,000 generations in our study. Although we cannot formally exclude the possibility that certain mutations in mismatch repair might increase fitness directly, it is much more likely that the evolved mutators we observed are deleterious or at best effectively neutral. Indeed, known mutator

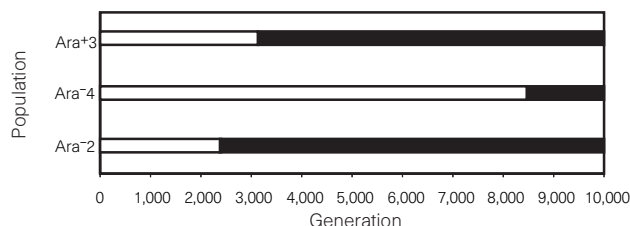


Figure 2 Time of appearance and evolutionary persistence of mutator phenotypes in experimental populations Ara⁻2, Ara⁻4 and Ara⁺3. White bars, wild type; black bars, mutator.

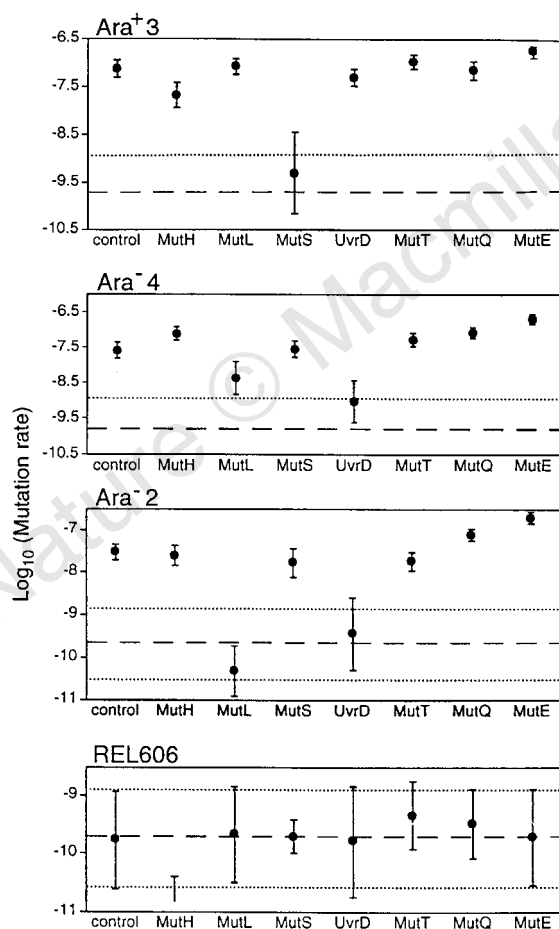


Figure 3 Mutation rates to nalidixic-acid resistance in 10,000-generation isolates from populations Ara⁺3, Ara⁻4 and Ara⁻2 and ancestor REL606 transformed with plasmids bearing wild-type alleles of seven known general mutator loci. Controls shown are plasmid free. Error bars give approximate 95% confidence limits. Only an upper confidence limit is shown for the case in which no mutants were obtained in any culture. For visual comparison, the dashed horizontal line and dotted horizontal lines in each panel illustrate the mutation rate and approximate 95% confidence limits, respectively, measured in the ancestral control.

alleles of *mutS*, *mutL* and *uvrD* do not increase cell fitness under our experimental conditions (C. Zeyl, unpublished data).

A prediction of the simulations conducted by Taddei *et al.*⁴ is that mutators will hitch-hike in some, but not all, finite asexual populations undergoing adaptation. Our experimental observations are consistent with this prediction. A further prediction is that fitness will be higher on average in mutator populations than in those that retain the wild-type mutation rate. However, this fitness effect is very small and subtle: approximately half of the mutator populations in the simulations of Taddei *et al.* did not show higher fitness, and the observed increases in fitness over contemporaneous wild-type populations were slight (approximately 1%) in the remainder (see Fig. 3b in ref. 4). We tested for a relationship between mutability and fitness in our experimental populations in several ways, none of which gave statistical significance. For example, we tested for a correlation between mutability ranks (as calculated from data obtained in screens for mutator phenotypes; see Methods) and relative fitness ranks averaged across the duration of the experiment in all 12 populations. The results were inconclusive ($r = 0.28$, $n = 12$, one-tailed $P = 0.19$). It is clear from our experiments and the simulations of Taddei *et al.*⁴ that increased mutation rate and not increased fitness is the more striking consequence of the hitch-hiking process.

Our finding that asexual populations can evolve high mutation rates in a relatively benign environment may help to explain observations associating mismatch repair mutators with certain cancers¹⁰ and with pathogenicity in *E. coli* and *Salmonella*⁹. As in our experimental populations, high mutation rates may evolve as a chance byproduct of adaptation in clonal tumour lineages and in populations of asexual pathogens^{17,18}. The potential for faster evolution once high mutation rates have evolved may have important health implications. □

Methods

Experimental system. The common ancestral strain was REL606, an Ara⁻ clone of *E. coli* B obtained by B. R. Levin from S. Lederberg¹⁹. A spontaneous Ara⁺ revertant was selected from REL606 and designated REL607. Six clones each from REL606 and REL607 were used to found the 12 experimental populations, which were propagated at 37 °C by daily 100-fold dilutions into 10 ml of fresh Davis minimal medium²⁰ supplemented with glucose at 25 µg ml⁻¹. Population sizes fluctuated between approximately 5×10^6 cells after dilution and 5×10^8 cells at the stationary phase. Isolates from each population were stored at -80 °C at 100-generation intervals during the first 2,500 generations of the experiment, and at 500-generation intervals thereafter.

Mutation rate measurements. Fluctuation tests²¹ to estimate a given rate of mutation were conducted simultaneously on all strains to provide a controlled comparison. Before testing, strains were revived and regrown for 3 days in the original experimental medium to re-establish the physiological conditions that had prevailed in the evolving populations. In general, 24 or more cultures of a given strain were grown from inocula of approximately 1,000 cells for each fluctuation test. For a given mutation rate measurement, all strains to be compared were grown separately in the same batch of Davis minimal medium. Cultures were grown to stationary phase before selective plating. (An additional 24 h in stationary phase had no discernible effect on the mutation rate in pilot studies.) Final population sizes were estimated by growing and sampling three extra cultures taken at random for each strain and measuring cell densities using a Coulter particle counter; the means were used in mutation rate calculations. Ara⁺ mutants were enumerated on Davis minimal agar medium supplemented with 4 mg ml⁻¹ arabinose; Nal^r mutants were enumerated on LB agar medium²² containing 20 µg ml⁻¹ of nalidixic acid; T5^r mutants were enumerated by plating cultures with excess phage T5 in soft agar.

Time of origin and persistence of mutator phenotypes. Mutator screens were conducted at approximately 500-generation intervals over the 10,000 generations of the experiment. Overnight cultures in 5 ml of Davis minimal medium (supplemented with 1,000 µg ml⁻¹ glucose) were assayed for Nal^r mutant numbers at the stationary phase. Expected distributions of mutants, based on mutator and ancestral mutation rates, were used to generate criteria

by which isolates could be scored as mutator or non-mutator with >95% confidence.

Complementation tests. Strains were transformed with plasmids carrying wild-type alleles for *mutH* (plasmid pGW1899)²³, *mutL* (pGW1842)²³, *mutS* (pGW1811)²³, *uvrD* (pGT26)²⁴, *mutT* (pSK25)²⁵, *dnaQ* (pMM5)²⁶ and *dnaE* (pMK9)²⁷, according to a standard protocol²⁸. Fluctuation tests were conducted as described above, except that all strains were propagated in LB medium (containing 60 µg ml⁻¹ of ampicillin where the strain was plasmid bearing), and five parallel cultures were used per fluctuation test.

Analysis of fluctuation test data. A local computer program using a Luria–Delbrück distribution-generating algorithm²⁹ was used to calculate maximum-likelihood mutation rates from fluctuation test data. Approximate 95% confidence intervals for the mutation rates illustrated in Fig. 1 were calculated from formulae³⁰. Approximate 95% confidence limits for the mutation rates illustrated in Fig. 3 are based on the theoretical variance of the maximum-likelihood estimate of $\ln(m)$, assuming normality, where m is the expected number of mutations per culture.

Received 3 March; accepted 6 May 1997.

- Drake, J. W. Spontaneous mutation. *Annu. Rev. Genet.* **25**, 125–146 (1991).
- Leigh, E. G. Natural selection and mutability. *Am. Nat.* **104**, 301–305 (1970).
- Ishii, K., Matsuda, H., Iwasa, Y. & Sasaki, A. Evolutionarily stable mutation rate in a periodically changing environment. *Genetics* **121**, 163–174 (1989).
- Taddei, F. *et al.* Role of mutator alleles in adaptive evolution. *Nature* **387**, 700–702 (1997).
- Cox, E. C. & Gibson, T. C. Selection for high mutation rates in chemostats. *Genetics* **77**, 169–184 (1974).
- Chao, L. & Cox, E. C. Competition between high and low mutating strains of *Escherichia coli*. *Evolution* **37**, 125–134 (1983).
- Tröbner, W. & Piechocki, R. Competition between isogenic *mutS* and *mut⁺* populations of *Escherichia coli* K12 in continuously growing cultures. *Mol. Gen. Genet.* **198**, 175–176 (1984).
- Mao, E. F., Lane, L., Lee, J. & Miller, J. H. Proliferation of mutators in a cell population. *J. Bacteriol.* **179**, 417–422 (1997).
- LeClerc, J. E., Li, B., Payne, W. L. & Cebula, T. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. *Science* **274**, 1208–1211 (1996).
- Modrich, P. Mismatch repair, genetic stability and tumour avoidance. *Phil. Trans. R. Soc. Lond. B* **347**, 89–95 (1995).
- Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am. Nat.* **138**, 1315–1341 (1991).
- Lenski, R. E. & Travisano, M. Dynamics of adaptation and diversification: A 10,000-generation experiment with bacterial populations. *Proc. Natl Acad. Sci. USA* **91**, 6808–6814 (1994).
- Vasi, F., Travisano, M. & Lenski, R. E. Long-term experimental evolution in *Escherichia coli*. II. Changes in life-history traits during adaptation to a seasonal environment. *Am. Nat.* **144**, 432–456 (1994).
- Travisano, M. & Lenski, R. E. Long-term experimental evolution in *Escherichia coli*. IV. Targets of selection and the specificity of adaptation. *Genetics* **143**, 15–26 (1996).
- Elena, S. F., Cooper, V. S. & Lenski, R. E. Punctuated evolution caused by selection of rare beneficial mutations. *Science* **272**, 1802–1804 (1996).
- Modrich, P. Mechanisms and biological effects of mismatch repair. *Annu. Rev. Genet.* **25**, 229–253 (1991).
- Nowell, P. C. The clonal evolution of tumor cell populations. *Science* **194**, 23–28 (1974).
- Moxon, E. R., Rainey, P. B., Nowak, M. A. & Lenski, R. E. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr. Biol.* **4**, 24–33 (1994).
- Lederberg, S. Genetics of host–control restriction and modification of deoxyribonucleic acid in *Escherichia coli*. *J. Bacteriol.* **91**, 1029–1036 (1966).
- Carlton, B. C. & Brown, B. J. In *Manual of Methods for General Bacteriology* (ed. Gerhardt, P.) 222–242 (American Society for Microbiology, Washington DC, 1981).
- Luria, S. E. & Delbrück, M. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28**, 491–511 (1943).
- Miller, J. H. *A Short Course in Bacterial Genetics* (Cold Spring Harbor Laboratory Press, NY, 1992).
- Pang, P. P., Lundberg, A. S. & Walker, G. C. Identification and characterization of the *mutL* and *mutS* gene products of *Salmonella typhimurium* LT2. *J. Bacteriol.* **163**, 1007–1015 (1985).
- Taucher-Scholz, G. & Hoffman-Berling, H. Identification of the gene for DNA helicase II of *Escherichia coli*. *Eur. J. Biochem.* **137**, 573–580 (1983).
- Bhatnagar, S. K. & Bessman, M. J. Studies on the mutator gene, *mutT*, of *Escherichia coli*. Molecular cloning of the gene, purification of the gene product, and identification of a novel nucleoside triphosphatase. *J. Biol. Chem.* **263**, 8953–8957 (1988).
- Horiuchi, T., Maki, H., Maruyama, M. & Sekiguchi, M. Identification of the *dnaQ* gene product and location of the structural gene for RNase H of *Escherichia coli* by cloning of the genes. *Proc. Natl Acad. Sci. USA* **78**, 3770–3774 (1981).
- Schaaper, R. M. & Cornacchio, R. An *Escherichia coli dnaE* mutation with suppressor activity toward mutator *mutD5*. *J. Bacteriol.* **174**, 1974–1982 (1992).
- Sambrook, E. F., Fritsch, T. & Maniatis, J. *Molecular Cloning: A Laboratory Manual* 2nd edn (Cold Spring Harbor Laboratory Press, NY, 1989).
- Ma, W. T., Sandri, G. v. H. & Sarkar, S. Analysis of the Luria–Delbrück distribution using discrete convolution powers. *J. Appl. Prob.* **29**, 255–267 (1992).
- Stewart, F. M. Fluctuation tests: how reliable are the estimates of mutation rates? *Genetics* **137**, 1139–1146 (1994).

Acknowledgements. We thank F. Taddei for sharing data before publication; C. Zeyl for permission to cite unpublished data; A. White for help with statistical analyses; B. Bohannon, L. Ekunwe and P. Frank for technical assistance; T. Cebula, S. F. Elena, D. G. MacPhee, J. Mongold and M. Travisano for discussions; and H. Maki and J. E. LeClerc for plasmids. Supported by the US NSF and by the Center for Microbial Ecology, Michigan State University.

Correspondence and requests for materials should be addressed to P.D.S. (e-mail: paulsnie@sas.upenn.edu).

Evidence from Turner's syndrome of an imprinted X-linked locus affecting cognitive function

D. H. Skuse*, R. S. James†, D. V. M. Bishop‡, B. Coppin§, P. Dalton†, G. Aamodt-Leeper*, M. Bacarese-Hamilton*, C. Creswell*, R. McGurk* & P. A. Jacobs†

* Behavioural Sciences Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

† Wessex Regional Genetics Laboratory, Salisbury District Hospital, Salisbury, Wiltshire SP2 8BJ, UK

‡ MRC Applied Psychology Unit, 15 Chaucer Road, Cambridge CB2 2EF, UK

§ Wessex Regional Genetics Service, Princess Anne Hospital, Coxford Road, Southampton SO16 5YA, UK

Turner's syndrome is a sporadic disorder of human females in which all or part of one X chromosome is deleted¹. Intelligence is usually normal² but social adjustment problems are common³. Here we report a study of 80 females with Turner's syndrome and a single X chromosome, in 55 of which the X was maternally derived (45,X^m) and in 25 it was of paternal origin (45,X^p). Members of the 45,X^p group were significantly better adjusted, with superior verbal and higher-order executive function skills, which mediate social interactions⁴. Our observations suggest that there is a genetic locus for social cognition, which is imprinted⁵ and is not expressed from the maternally derived X chromosome. Neuropsychological and molecular investigations of eight females with partial deletions of the short arm of the X chromosome⁶ indicate that the putative imprinted locus escapes X-inactivation⁷, and probably lies on Xq or close to the centromere on Xp. If expressed only from the X chromosome of paternal origin, the existence of this locus could explain why 46,XY males (whose single X chromosome is maternal) are more vulnerable to developmental disorders of language and social cognition, such as autism, than are 46,XX females⁸.

An increasing number of mammalian genes are known to be subject to genomic imprinting, defined as parental origin-specific differential gene expression⁵. No imprinted gene has yet been described on the X chromosome in humans⁹, although the *Xist* gene has been shown to be imprinted in the mouse¹⁰. We considered that it should be possible to identify the effects of an X-linked imprinted locus by comparing classes of females with Turner's syndrome. In this chromosomal disorder all, or a substantial part, of one X chromosome is missing as a result of non-disjunction (chromosome loss during gametogenesis or early cleavage of the zygote). In 70% of monosomic (45,X) Turner-syndrome females, the single X chromosome is maternal in origin¹; in the remainder it is paternal. Normal females (46,XX) possess both a maternally derived X chromosome (X^m) and a paternally derived X chromosome (X^p), one of which is randomly inactivated in any given somatic cell⁷. In monosomy X, the single chromosome is never inactivated. Differences in physical or behavioural phenotype between 45,X^p and 45,X^m Turner-syndrome subjects might therefore indicate the existence of an imprinted genetic locus.

Impaired social competence and adjustment are frequent in Turner's syndrome³, but a minority have good social skills¹¹. Intelligence is usually normal in monosomic (45,X) cases². We wished to test the hypothesis that 45,X^p females would be distinguishable from 45,X^m females by their social behaviour.

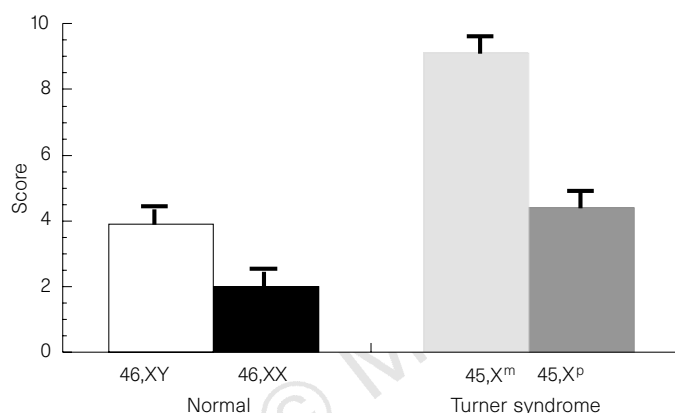
We karyotyped 80 monosomic (45,X) females and eight with deletions of the short arm of one X chromosome (46,XXp-). The

Box 1 Scale measuring social cognition

Complete the following section by circling 0 if the statement is not at all true of your child, 1 if it is quite or sometimes true of your child, and 2 if it is very or often true of your child:

- * lacking an awareness of other people's feelings
- * does not realise when others are upset or angry
- * is oblivious to the effect of his/her behaviour on other members of the family
- * behaviour often disrupts normal family life
- * very demanding of people's time
- * difficult to reason with when upset
- * does not seem to understand social skills: e.g., interrupts conversation
- * does not pick up on body language
- * unaware of acceptable social behaviour
- * unknowingly offends people with behaviour
- * does not respond to commands
- * has difficulty following commands unless they are carefully worded

Internal consistency for set of 12 questions: Standardised item alpha 0.94.



parental origin of the normal X chromosome was determined by comparing proband and parental DNA polymorphisms located on distal Xp, in a region that was deleted in both 45,X and 46,XXp-patients. Of the 80 45,X females, 25 were 45,X^p and 55 were 45,X^m, with ages from 6 to 25 years. Clinical records did not reveal any significant group differences in terms of physical phenotype or in the provision of hormone-replacement therapy¹². From a first-stage screening survey¹³ of parents and teachers, using standardized instruments^{14–16}, we discovered that 40% of 45,X^m girls of school age had received a statement of special educational needs, indicating academic failure, compared with 16% of 45,X^p subjects ($P < 0.05$); the figure in the general population is just 2%. We also found that clinically significant social difficulties affected 72.4% of the 45,X^m subjects over 11 years of age (21 of 29), compared with 28.6% of 45,X^p females (4 of 14) ($P < 0.02$).

Such phenotypic variability between the two classes of monosomy X subjects could indicate the existence of an imprinted genetic locus, at which gene(s) that influence social adjustment are expressed only from the paternally derived X chromosome. On the maternally derived X chromosome, the corresponding locus would be silenced. This could account for the excess of social and learning difficulties among 45,X^m females compared with the 45,X^p variant. Pilot interviews and observations showed that 45,X^m females in particular lacked flexibility and responsiveness in social interactions. We therefore devised a questionnaire relevant to social cognition to summarize the main features of their behaviour (Box 1). Parents of our sample of Turner's syndrome females and the parents of age-matched normal male and female comparisons

Table 1 Neuropsychological test results

	Turner's syndrome		Normal	
	45,X ^m	45,X ^p	46,XX	46,XY
	(mean ± s.d.)	(mean ± s.d.)	(mean ± s.d.)	(mean ± s.d.)
IQ				
Verbal	96.2 ± 15.9	106.4 ± 14.4	100.1 ± 16.7	98.6 ± 17.1
Non-verbal	79.5 ± 18.8	82.1 ± 15.9	–	–
Executive function tasks				
Behavioural inhibition	8.7 ± 7.1	5.7 ± 4.8	5.3 ± 4.1	6.8 ± 3.4
Planning ability	6.1 ± 1.7	7.4 ± 1.7	6.6 ± 1.6	7.2 ± 1.4

The 45,X^p females have significantly higher verbal IQ than 45,X^m subjects ($P < 0.02$), but neither Turner group differs significantly from the normal female comparisons. Non-verbal IQ was measured only in Turner-syndrome subjects and does not significantly distinguish the subgroups; it incorporates tests of visuospatial abilities, which are known to be specifically impaired in this condition²⁹. In all analyses using the executive function measures, age has been covaried because, unlike conventional IQ measures, these tests are not yet standardized for age. Behavioural inhibition scores (Same–Opposite World) are measured in seconds, higher scores indicating more difficulty completing the task accurately. The 45,X^m females are less competent than either 45,X^p subjects ($P < 0.02$) or normal females ($P < 0.03$). Males are less competent than normal females ($P < 0.03$). On the planning task (Tower of Hanoi), neither the two Turner subgroups nor normal males and females are significantly distinguished from one another by the mean highest level achieved.

Figure 1 Subscale scores (mean + s.e.) of questionnaire on social-cognitive impairment (Box 1). Higher scores indicate poorer social cognitive skills. The 45,X^m Turner-syndrome females score higher than 45,X^p females and both normal groups ($P < 0.0001$). Normal males score higher than normal females ($P < 0.001$); the effect size of this difference is 0.58, implying that the upper 50% of females score higher than approximately 72% of males. The ratios of mean social-dysfunction scores male:female and 45,X^m:45,X^p are very similar 2.2:1 and 2.1:1, respectively). The overall higher scores for the Turner-syndrome subjects, compared with normal females, may reflect the contribution made by visuospatial abilities to social cognition³¹. These abilities are impaired equally in both monosomic groups. No information regarding parental origin of the normal X chromosome was made available to parents, their consultants, or members of the research team gathering these or other data.

completed the questionnaire. The results for subjects aged from 6 to 18 years of age confirm there are significant differences between 45,X^m and 45,X^p females in the predicted direction (Fig. 1). We found that 45,X^m subjects obtained significantly higher scores than 45,X^p females on our measure of social-cognitive dysfunction. Normal boys also obtained significantly higher scores on the questionnaire than did normal girls, indicating poorer social cognition (Fig. 1). The magnitude and direction of this difference are compatible with the hypothesis that there is an imprinted locus on the X chromosome that influences the development of social cognitive skills (although the finding is of course also compatible with other explanations of gender differences in behaviour¹⁷). If the putative locus only expressed a gene (or genes) from the X chromosome of paternal origin (X^p), we would expect a tendency for normal females to have superior social cognitive skills than males. Because males (46,XY) invariably inherit their single X chromosome from their mothers, the genetic locus would be silenced. In contrast, the gene(s) would be expressed from X^p in approximately half of the cells of normal females if it were inactivated, and from all cells if it escaped X-inactivation⁷.

We hypothesized that an imprinted X-linked locus, either without a Y-linked homologue or with a Y homologue showing a lower level of expression, could also explain why males are markedly more vulnerable than females to pervasive developmental disorders affecting social adjustment and language, such as autism⁸. Accordingly, 45,X^m Turner females should be exceptionally vulnerable to disorders of language and social adjustment that are usually more common in males. Consistent with this hypothesis, we identified

Table 2 Cytogenetic and molecular information

Patient	Karyotype	Breakpoints between	
		proximal	distal
85/5142	46,X,del(X)(qter → p22:)	DXS8036	DXS1053
95/4894	46,X,del(X)(qter → p21.2:)	DXS1036	DXS985
95/4247	45,X[33]/46,X,del(X)(qter → p21.2:)[17]	DXS1036	DXS985
95/3557	45,X[32]/46,del(X)(qter → p22.11:)[68]	DXS8039	DXS8049
91/219	46,X,del(X)(qter → p11:)	DXS1208	DXS1055
96/863	46,X,del(X)(qter → 11.2:)	DXS8062	DXS1003
96/8266	46,X,del(X)(qter → p11.23:)	DXS423E	UBE1
96/5509	45,X[9]/46,X,der(X)del(X)(p21.2 → pter)inv(X)(p21.2 q22.1)[166]	DXS1067	DXS8039

Cytogenetic analysis was performed by standard techniques and the chromosomes were examined in a minimum of 100 cells. To map the position of the breakpoints in the deleted X chromosomes, DNA from probands and parents was tested using PCR amplification of polymorphic microsatellite repeat sequences located along the length of Xp. For DXS423E and UBE1, the status of the locus in the proband was determined by fluorescence *in situ* hybridization (FISH) using metaphase chromosome spreads. The breakpoints lie between the most distal non-deleted marker and the most proximal deleted marker.

three Turner-syndrome females with autism (meeting ICD-10 criteria)¹⁸, out of the total unbiased sample assessed personally in the course of this investigation (3.75%). All three females had retained a normal maternal X chromosome. The population prevalence of autism is less than 1 per 10,000 females⁸.

We then sought corresponding differences in the results of neuropsychological testing of both normal and Turner-syndrome subjects. We found that 45,X^P females were significantly superior in verbal intelligence to 45,X^m females (Table 1). Verbal IQ was negatively correlated with the social dysfunction score in the sample as a whole ($r = -0.41$, $P < 0.002$). Both monosomic Turner subgroups were equivalently impaired in non-verbal (including visuospatial) abilities.

We subsequently performed several more focused neuropsychological assessments. First, although we had found that verbal abilities were moderately good predictors of social cognition, we hypothesized that higher-order cognitive skills would be better predictors. These are not measured directly by conventional intelligence tests. The executive functions of the prefrontal cortex⁴ exert an important influence on social interactions, and include skills that allow for the development of strategies of action and the inhibition of distracting impulses when striving towards a goal. Developmental disorders of social adjustment and language are associated with impairment in measures of executive function¹⁹. Many monosomic Turner-syndrome subjects have executive-function deficits². We also predicted that 45,X^P females would perform better than 45,X^m females in tests of executive function skills. Finally, we predicted that abilities in which 45,X^P subjects were superior to 45,X^m subjects would also distinguish normal females from males, and in the same direction.

We chose tests of both planning ability (Tower of Hanoi) and behavioural inhibition (Same/Opposite World). The results were consistent with the initial hypothesis. Although the social-cognitive impairment score correlated independently with verbal IQ and with both measures of executive function, only planning ability ($r = -0.4$; $P < 0.006$) and behavioural inhibition ($r = -0.37$; $P < 0.015$) retained significance when the three variables were forced into a multiple regression analysis. The second prediction was partly confirmed: there were significant differences between 45,X^P and 45,X^m subjects in terms of the behavioural inhibition task, although not on the test of planning ability. The third prediction was fully confirmed (Table 1): there was no significant difference in the highest level achieved on the planning task between normal males and females. However, on the behavioural-inhibition task, normal females were superior to males and their mean scores were very similar to those of the 45,X^P subjects. Previous reports have noted gender differences on inhibition tasks, and they have been conceptually linked to corresponding differences in social behaviour²⁰.

We then attempted to map the putative imprinted locus, provisionally, by studying eight females, ascertained as part of the Turner-syndrome project, who had large terminal deletions of the short arm

of the paternally derived X chromosome (a 46,X^m X^P p-karyotype). These deletions all extended to a point proximal to the *MLS* gene at Xp22.3 (Table 2); the paternal X chromosome was consequently preferentially inactivated⁶. On examination, no-one in this series was found to have any significant learning difficulties. The mean verbal intelligence score for the group was 103.4 (s.d., 13.7), and their mean non-verbal IQ was 93 (s.d., 11.2). Their mean social dysfunction score was 3.75 (s.d., 3.1), a value very similar to that of the 45,X^P subjects (Fig. 1). We drew the following conclusions from these data. First, the imprinted locus had not been deleted on the structurally abnormal paternal X chromosome, and so it must lie on Xq or on Xp closer to the centromere than, *UBE1* at Xp11.23. Second, the imprinted locus was not subject to X-inactivation, or the preferentially inactivated, partly deleted chromosome would not have expressed it. We already know of several genes that escape X-inactivation²¹.

We expected that isochromosomes of the long arm of the X chromosome [i(Xq)] would help in the mapping of the putative imprinted locus. However, virtually all i(Xq) chromosomes have been shown to contain proximal Xp sequences²². Duplication and consequent trisomy of the long arm of the X chromosome further complicate the interpretation of correlations between phenotype and genotype. Accordingly, this approach did not provide unambiguous evidence to assist in the deletion mapping of the locus.

An imprinted locus is not the only possible explanation for our findings. Among the 45,X^P females, there may have been a greater degree of cryptic mosaicism (with a normal 46,XX cell line) than among those who were 45,X^m. Some degree of mosaicism in apparently monosomic females may be essential for the fetus to avoid spontaneous abortion²³. We examined both blood and cheek cells, tissues of mesodermal and ectodermal origin, respectively, and found two cryptic mosaics, but both were from the 45,X^m group.

Males are substantially more vulnerable to a variety of developmental disorders of speech, language impairment and reading disability, as well as more severe conditions such as autism⁸. Our findings are consistent with the hypothesis that the locus described, which we propose to be silent both in males and 45,X^m females, acts synergistically with susceptibility loci elsewhere on the genome to increase the male-to-female prevalence ratio of such disorders. Our data on normally developing children suggest it may also exert an effect on social and cognitive abilities in the normal range. These preliminary findings could thus provide evidence for the evolution of an imprinted X-linked locus that underlies the development of sexual dimorphism in social behaviour¹⁷. □

Method

Subjects. This study, which was approved by the local hospital ethics committee, involved 88 females with Turner's syndrome (80 monosomic and 8 partial X-chromosome deletions; age range, 6–25 years). They were selected from a national survey of Turner's syndrome and from records of the Wessex Regional Laboratory. The mean age of the 45,X^m females was 162.3 months (s.d., 57.6), that of the 45,X^P females was 164.5 months (s.d., 57.7), and that of

the Xp- females was 185.8 months (s.d., 74.9). All subjects were healthy, with no significant neurological disease. Females with an Xp- chromosome were all referred for investigation because of short stature in middle childhood, with one exception who was karyotyped at birth. Neuropsychological test results are presented for subjects with verbal IQs ≥ 65 (three 45,X^m subjects and one 45,X^p subject had verbal IQs that fell out of range). Parents rated 70 normal males and 71 normal females (age range, 6–18 years) on the social-cognition scale. The neuropsychological test battery was used to assess 68 normal males and 91 normal females (age range, 6–25 years). Verbal IQs were in the range 65–151. All normal comparison subjects were recruited from urban and suburban schools (6–18 years) and from hospital staff (18–25 years).

Behavioural and cognitive measures. Initial screening was conducted by postal questionnaires using a well-standardized set of instruments^{14–16}. These were completed by parents, teachers and the Turner-syndrome subjects themselves (11 years and over). The social cognition questionnaire (Box 1) was completed by parents only. In a survey of 175 Turner-syndrome subjects for whom we obtained parental ratings on two occasions, a mean of 2.7 years apart, the intraclass correlation coefficient was 0.81 ($P < 0.01$). Scores correlate with the self-rated social problem subscale of the YSR¹⁶ 0.58 ($P < 0.002$), with the teacher rating on the TRF¹⁵ 0.54 ($P < 0.001$), and with the parent-rated CBCL¹⁴ 0.69 ($P < 0.001$). The range of scores was 0–23 in the Turner-syndrome sample and 0–21 in the normal sample (maximum score of 24). The CBCL¹⁴ was completed by 70 parents, the YSR¹⁶ was completed by 40 subjects over 11 years of age, and the TRF¹⁵ was completed by 45 teachers. Clinical significance of social problems was estimated according to clinical *T* scores^{14–16}. Measures of cognition included the Wechsler Intelligence Scales for Children (WISC III-UK)²⁴ and the Wechsler Adult Intelligence Scales-Revised (WAIS-R)²⁵. The behavioural inhibition task was the Same-Opposite World subtest from the Test of Everyday Attention for Children²⁶. This yields a time measure that ascertains the difference in latency for a subject responding to a series of stimuli on a task of sequential responses, which are named both as they appear and then opposite to their appearance. The subject reads a random series of numbers (1 and 2) saying 'one' to 1, and 'two' to 2. The subjects then repeat the task on a new series, but this time they have to inhibit the prepotent response and instead say 'two' to 1, and 'one' to 2, correcting any errors before proceeding. Test-retest reliability on a sample of 70 normal children gave an intraclass correlation coefficient of 0.62 ($P < 0.001$). The Tower of Hanoi task was based on the procedure described previously²⁷. It was scored according to the most complex level of the problem the child could solve reliably. Test-retest reliability gave an intraclass correlation coefficient for the highest level achieved of 0.45 ($P < 0.001$), which is in line with expectations for a test that makes novel demands of this nature²⁸.

Received 17 February; accepted 1 May 1997.

- Jacobs, P. A. *et al.* A cytogenetic and molecular reappraisal of a series of patients with Turner's syndrome. *Ann. Hum. Genet.* **54**, 209–223 (1990).
- Pennington, B. F. *et al.* The neuropsychological phenotype in Turner syndrome. *Cortex* **21**, 391–404 (1985).
- McCauley, E., Ito, J. & Kay, T. Psychosocial functioning in girls with the Turner syndrome and short stature. *J. Am. Acad. Child Psychiat.* **25**, 105–112 (1986).
- Damasio, A. R. On some functions of the human prefrontal cortex. *Proc. N. Y. Acad. Sci.* **769**, 241–251 (1995).
- Barlow, D. P. Gametic imprinting in mammals. *Science* **270**, 1610–1613 (1995).
- Ballabio, A. & Andria, G. Deletions and translocations involving the distal short arm of the human X chromosome: review and hypotheses. *Hum. Mol. Genet.* **1**, 221–227 (1995).
- Lyon, M. F. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* **190**, 372–373 (1961).
- Bailey, A., Phillips, W. & Rutter, M. Autism: towards an integration of clinical, genetic, neuropsychological and neurobiological perspectives. *J. Child Psychol. Psychiat.* **37**, 89–126 (1996).
- Ledbetter, D. H. & Engel, E. Uniparental disomy in humans: development of an imprinting map and its implications for prenatal diagnosis. *Hum. Mol. Genet.* **4**, 1757–1764 (1995).
- Zuccotti, M. & Monk, M. Methylation of the mouse *Xist* gene in sperm and eggs correlates with imprinted *Xist* expression and paternal X-inactivation. *Nature Genet.* **9**, 316–320 (1995).
- McCauley, E., Kay, T., Ito, J. & Trader, R. The Turner syndrome: cognitive deficits, affective discrimination and behaviour problems. *Child Dev.* **58**, 464–473 (1987).
- Saenger, P. Clinical Review 48: The current status of diagnosis and therapeutic intervention in Turner's syndrome. *J. Clin. Endocrinol. Metabol.* **77**, 297–301 (1993).
- Skuse, D., Percy, E. L. & Stevenson, J. in *Growth, Stature, and Adaptation. Behavioral, Social, and Cognitive Aspects of Growth Delay* (eds Stabler, B. & Underwood, L.) 151–164 (UCP, Chapel Hill, 1994).
- Achenbach, T. M. *Manual for the Child Behavior Checklist/4-18 and 1991 Profile* (Department of Psychiatry, University of Vermont, Burlington, VT, 1991).
- Achenbach, T. M. *Manual for the Teacher's Report Form and 1991 Profile* (Department of Psychiatry, University of Vermont, Burlington, VT, 1991).
- Achenbach, T. M. *Manual for the Youth Self-Report Form and 1991 Profile* (Department of Psychiatry, University of Vermont, Burlington, VT, 1991).

- Eagley, A. H. The science and politics of comparing men and women. *Am. Psychol.* **50**, 145–158 (1995).
- World Health Organization *The ICD-10 Classification of Mental and Behavioural Disorders: Clinical Descriptions and Diagnostic Guidelines* (World Health Organization, Geneva, 1992).
- Pennington, B. F. & Ozonoff, S. Executive functions and developmental psychopathology. *J. Child Psychol. Psychiat.* **37**, 51–87 (1996).
- Bjorklund, D. F. & Kipp, K. Parental investment theory and gender differences in the evolution of inhibition mechanisms. *Psychol. Bull.* **120**, 163–188 (1996).
- Disteche, C. M. Escape from X inactivation in human and mouse. *Trends Genet.* **11**, 17–22 (1995).
- Wolff, D. J., Miller, A. P., Van Dyke, D. L., Schwartz, S. & Willard, H. F. Molecular definition of breakpoints associated with human Xq isochromosomes: implications for mechanism of formation. *Am. J. Hum. Genet.* **58**, 154–160 (1996).
- Hassold, T., Pettay, D., Robinson, A. & Uchida, I. Molecular studies of parental origin and mosaicism in 45,X conceptuses. *Hum. Genet.* **89**, 647–652 (1992).
- Wechsler, D. *Wechsler Intelligence Scale for Children 3rd UK edn* (Psychological Corporation, London, 1992).
- Wechsler, D. *Wechsler Adult Intelligence Scales-Revised* (Psychological Corporation, New York, 1986).
- Borys, S. V., Spitz, H. H. & Dorans, B. A. Tower of Hanoi performance of retarded young adults and nonretarded children as a function of solution length and goal state. *J. Exp. Child Psychol.* **33**, 87–110 (1982).
- Manly, T., Robertson, I. H. & Anderson, V. *The Test of Everyday Attention for Children (TEACH)* (Thames Valley Test Company, Bury St Edmunds, in the press).
- Rabbitt, P. M. A. in *Methodologies of Frontal and Executive Function* (ed. Rabbitt, P. M. A.) (Psychology Press, Hove, in the press).
- Temple, C. M. & Carney, R. A. Patterns of spatial functioning in Turner's syndrome. *Cortex* **31**, 109–118 (1995).

Acknowledgements. We thank E. Percy, S. Cave, A. O'Herlihy, R. South, J. Smith, M. Power and D. Robinson for assistance; M. Pembrey for comments and discussion; many paediatric consultants for assisting with the recruitment of patients, the schools who participated, and all of the subjects of our investigation and their families for their time. This research was supported by the Wellcome Trust and the Child Growth Foundation. Compilation of the national register of Turner syndrome was supported by the British Society for Paediatric Endocrinology and by Pharmacia.

Correspondence and requests for material should be addressed to D.H.S. (e-mail: dskuse@ich.ucl.ac.uk).

Molecular evidence for an ancient duplication of the entire yeast genome

Kenneth H. Wolfe & Denis C. Shields

Department of Genetics, University of Dublin, Trinity College, Dublin 2, Ireland

Gene duplication is an important source of evolutionary novelty^{1,2}. Most duplications are of just a single gene, but Ohno¹ proposed that whole-genome duplication (polyploidy) is an important evolutionary mechanism. Many duplicate genes have been found in *Saccharomyces cerevisiae*, and these often seem to be phenotypically redundant^{3–7}. Here we show that the arrangement of duplicated genes in the *S. cerevisiae* genome is consistent with Ohno's hypothesis. We propose a model in which this species is a degenerate tetraploid resulting from a whole-genome duplication that occurred after the divergence of *Saccharomyces* from *Kluyveromyces*. Only a small fraction of the genes were subsequently retained in duplicate (most were deleted), and gene order was rearranged by many reciprocal translocations between chromosomes. Protein pairs derived from this duplication event make up 13% of all yeast proteins, and include pairs of transcription factors, protein kinases, myosins, cyclins and pheromones. Tetraploidy may have facilitated the evolution of anaerobic fermentation in *Saccharomyces*.

We searched systematically for duplicated regions^{6,7} in the complete yeast genome⁸ by using BLASTP⁹ amino-acid sequence similarity searches of all yeast proteins against one another, and plotted the results on dot matrices. Duplicate regions are visible as a diagonal series of 'hits' with conserved gene orientation. In the example shown in Fig. 1, three separate diagonals indicate three distinct regional duplications between chromosomes X and XI. Within each region the homologues are interspersed with genes that are not now duplicated. We propose that this is the result of random deletion of individual duplicated genes from one or other chromosome subsequent to the initial duplication of the whole region.

In the whole genome, 55 duplicate regions were identified

containing 376 pairs of homologous genes (Fig. 2). Amino-acid sequence identity between the pairs ranges from 24% to 100%, with a mean of 63%. The criteria used to define a duplicate region were: (1) BLASTP high scores of ≥ 200 for each gene pair (these have an associated significance of $P = 10^{-18}$ or less); (2) at least three pairs of homologues with intergenic distances of ≤ 50 kilobases (kb) on each chromosome; and (3) conservation of gene order and orientation (with allowance for small inversions within some blocks). The extent of duplicated regions is significantly in excess of what would be expected by chance if homologous genes were distributed at random, as measured by two statistical tests (see Methods). The duplicated regions are on average 55 kb long and contain a mean of 6.9 duplicate gene pairs. Together they span 50% of the genome. This is a minimal estimate because we did not consider possible duplicated regions that contain just one or two gene pairs. In the 55 duplicate regions, 25% of the genes (743 of 2,905) are duplicated, and again this is a minimal estimate because some less well conserved duplicate genes (BLASTP < 200) may be present within the blocks. Many regions also contain duplicate tRNA genes at equivalent locations. The 376 pairs of duplicate proteins account for only 12% of all the yeast sequence pairs having a BLASTP score above 200 (after excluding yeast retrotransposon (Ty) element sequences); most of the others are hits among members of large families such as sugar permeases, protein kinases, the AAA superfamily and the proteins encoded by subtelomeric repeats. However, these 376 pairs located in duplicate regions account for 42% of all 'simple' duplicate gene pairs in yeast (that is, pairs of genes that are one another's only significant BLASTP hit).

How did these 55 duplicated regions arise? They were formed either by many successive independent duplications (each involving dozens of kilobases), or simultaneously by a single duplication of the entire genome (tetraploidy) followed by reciprocal translocations between chromosomes to produce a mosaic of duplicated blocks. A polyploid origin for the yeast genome was first proposed in 1987 by Smith⁵.

We favour the tetraploidy and translocation model for two reasons. First, for 50 of the 55 duplicate regions, the orientation of the entire block with respect to the centromere is the same in the two copies (Fig. 2), which is significantly non-random ($\chi^2_1 = 18$). Block orientations are expected to be conserved if the blocks were formed by reciprocal translocations among duplicate chromosomes, whereas if each block was made by an independent duplication its orientation should be random. Moreover, even the gene pairs outside the identifiable duplicated blocks (Fig. 2) show a bias towards conservation of transcriptional orientation with respect to the centromere. For 'simple' gene pairs (defined above) in which both genes lie outside the 55 blocks, most (117 out of 172 pairs) show conserved transcriptional orientation, whereas no bias of orientation is seen in pairs in which one of the genes lies in a block but the other does not. This suggests that our method may have overlooked numerous smaller duplicated blocks. For example, the large unassigned region on the right arm of chromosome X (Fig. 2) becomes paired with unassigned parts of chromosomes XVI, XIII, IV and VIII if the criterion for defining a block is reduced from three to two pairs of duplicated genes.

Second, based on a Poisson distribution of block sizes, 55 successive duplications would be expected to result in about seven triplicated regions (that is, duplicates of duplicates), but we observe none (or at most one; see Methods). This difference is highly significant. Together, these two statistical tests support a model of tetraploidy in which the regional duplications are relics of a whole-genome duplication.

The mosaic pattern of duplicated segments leads us to propose that *S. cerevisiae* is an ancient tetraploid, similar to maize¹⁰ and perhaps vertebrates¹¹. Our model for this is that two ancestral diploid yeast cells, each containing about 5,000 genes, fused to form a tetraploid. Depending on the species relationship of the two

cells, this could have been either autotetraploidy or allotetraploidy. This species then became diploid (underwent a decay of sequence identity), and most (about 85%) of the duplicate copies were deleted, leaving the current species with a haploid/diploid life cycle and 5,800 genes that include many duplicates. The original chromosome-sized duplications were then broken up into smaller blocks by reciprocal translocations. About half of the 800 duplicate gene pairs are shown in Fig. 2, and the remainder are presumed to lie in regions that have been fragmented too severely by translocation, deletion or transposition to be detectable by the methods used here. Autotetraploidy sometimes occurs in yeast by fusion between spontaneously arising diploids that are homozygous at the *MAT* locus¹², and recent allotetraploidy has been proposed for *S. carlsbergensis*¹³. Reciprocal translocation, which we propose to be the mechanism causing fragmentation of the duplicated blocks, has been observed in comparisons of chromosomes between *S. cerevisiae* and *S. bayanus*¹⁴. The arrangement of some sets of blocks, such as the juxtaposition of both copies of blocks 50 and 14 beside both copies of blocks 23 and 37 (Fig. 2), also implicates reciprocal translocation. This model of yeast genome evolution could be tested by comparison of gene order in related genera such as *Kluyveromyces* and *Candida*. A similar model in which most, but not all, of the original chromosomes were duplicated (aneuploidy) seems less likely but cannot be ruled out.

The closest relatives of yeast for which a substantial sequence data set is available are species in the genus *Kluyveromyces*¹⁵. Comparison of the gene sequences and gene order of *S. cerevisiae* and

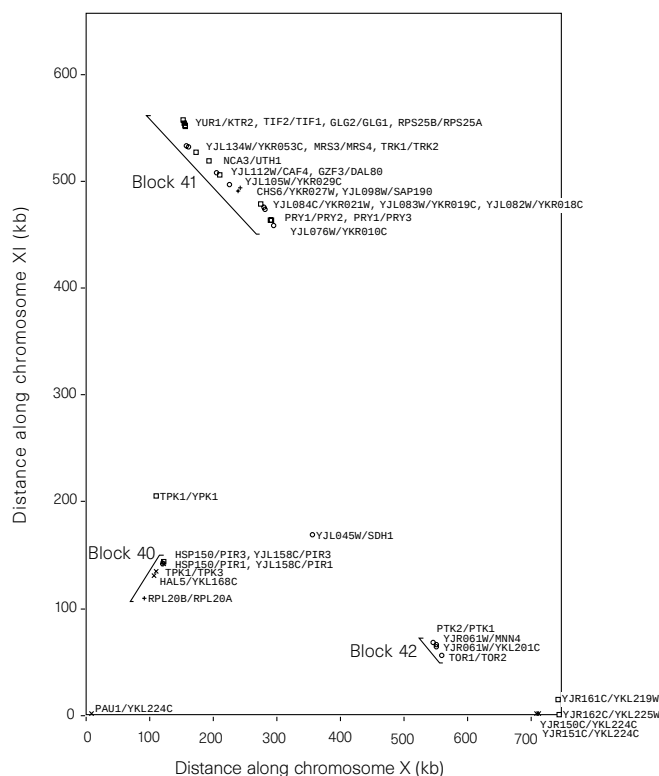
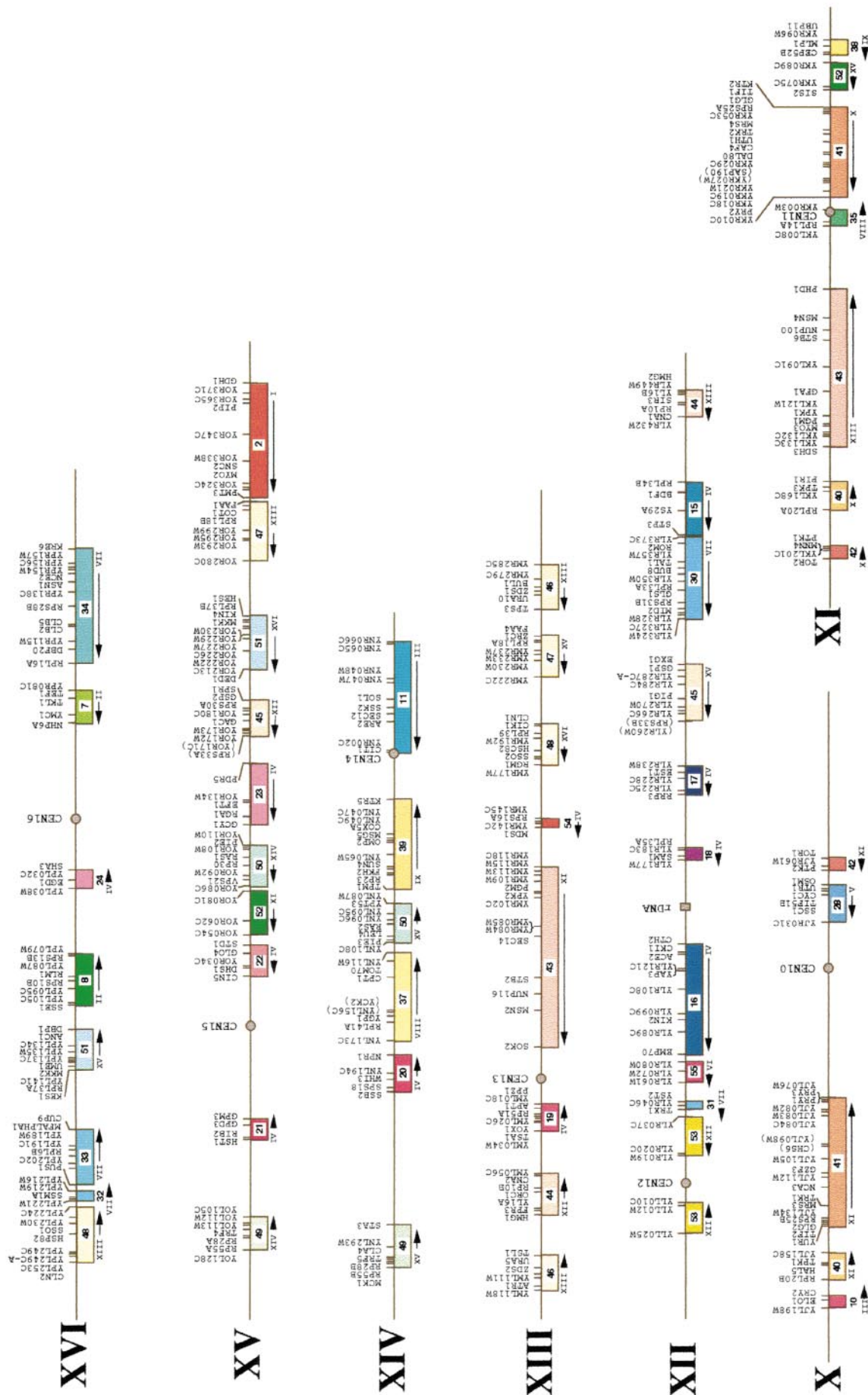
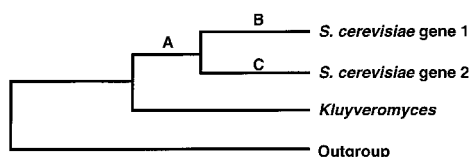


Figure 1 Locations of similar genes on chromosomes X and XI. All pairs of proteins with BLASTP scores ≥ 200 are plotted at the position of their genes on the two chromosomes. Ty elements have been omitted. Symbols indicate gene orientations: +, W (Watson strand; left-to-right transcription) on both chromosomes; $\times$, C (Crick strand; right-to-left transcription) on both; squares, C on chromosome X but W on chromosome XI; circles, W on chromosome X but C on chromosome XI. Three diagonals, corresponding to duplicated regions shown in Fig. 2, are marked.





from <http://acer.gen.tcd.ie/~khwolfe/yeast>.



Block	Gene 1 / Gene 2	Bootstrap support (%)	Age	90% Confidence interval	Amino-acid sites	Branch length	Outgroup
						A B C	
3	NTH2 / NTH1	100	0.70	(0.59 - 0.82)	714	0.052 0.131 0.115	Candida albicans
3	GAL1 / GAL3	99	0.55	(0.41 - 0.76)	468	0.100 0.115 0.130	Homo sapiens
37	YCK1 / YCK2	97	0.48		343	0.036 0.046 0.024	Sch. pombe
36	HXK1 / HXK2	94	0.77	(0.62 - 0.95)	484	0.037 0.147 0.107	Sch. pombe
45	EXG1 / SPR1	90	0.80	(0.67 - 0.97)	418	0.051 0.166 0.270	Candida albicans
10	CRY1 / CRY2	89	0.40		137	0.015 0.014 0.007	Homo sapiens
21	SIR2 / HST1	45	0.86	(0.66 - 1.00)	402	0.028 0.167 0.182	Caenorhabditis elegans
28	CYC7 / CYC1	34	0.82		106	0.017 0.110 0.057	Sch. pombe
30	YGR043C / TAL1	24	1.00		332	0.000 0.231 0.149	Homo sapiens
Genes with apparent gene conversion							
8	RPS10A / RPS10B	100	0.04		233	0.042 0.000 0.004	Sch. pombe
20	SSB1 / SSB2	100	0.06	(0.01 - 0.11)	613	0.049 0.002 0.004	Candida albicans
37	RPL41B / RPL41A	100	0.00		105	0.054 0.000 0.000	Candida tropicalis

Kluyveromyces suggests that these lineages diverged before the proposed genome duplication in *S. cerevisiae* (Fig. 3). This implies that the *Kluyveromyces* homologues of all of the genes named in Fig. 2 should be single-copy, and we did not find any examples to contradict this in the literature. *K. lactis* has a genome of 12 million base pairs, slightly smaller than that of *S. cerevisiae*, but has only six chromosomes. Two of its centromeres¹⁶ are orthologous to pairs of *S. cerevisiae* centromeres that are located in duplicated blocks 11 and 35.

Phylogenetic trees were drawn from protein sequences of 12 *S. cerevisiae* duplicate pairs with homologues in *Kluyveromyces* and an outgroup (Fig. 3). In nine of these there is strong bootstrap support ($\geq 89\%$) for a branching order that places the two *S. cerevisiae* sequences together; in the others there is no strong support for any order. We estimated the ages of the duplications in *S. cerevisiae* relative to the speciation between the two yeast species. Three gene pairs (*SSB1/SSB2* and two ribosomal proteins) yield very young ages, probably because they have been involved in recent gene conversions within *S. cerevisiae* (Fig. 3). Of the five sequences for which there are sufficient data to calculate a confidence interval by bootstrapping, and excluding *SSB1/SSB2*, the mean relative age of duplication is 0.74 (s.d. 0.12). The date of the divergence between *Saccharomyces* and *Kluyveromyces* is not known with any certainty, but assuming a constant molecular clock for 18S ribosomal RNA¹⁵ and an animal/fungal divergence time of 10^9 years, we estimate it to be roughly 1.5×10^8 years ago, which places the genome duplication roughly 10^8 years ago.

Our model of massive gene deletion in the wake of genome duplication predicts that some groups of genes that are adjacent in *Kluyveromyces* should have homologues in *S. cerevisiae* that are not themselves duplicated, but are located within different copies of duplicated blocks. This is the case with the genes *GAL4* (*LAC9*) and *SGS1*. In *S. cerevisiae* these genes are single-copy and are located on chromosomes XVI and XIII, respectively, in equivalent intervals within block 48 (between the pairs *HSP82/HSC82* and *YPL249C/YMR192W*; Fig. 2). In *K. lactis* *GAL4* (*LAC9*) and *SGS1* are neighbours (R. S. Keogh and K.H.W., unpublished data). This suggests that *GAL4* and *SGS1* were originally adjacent in *Saccharomyces*, but after the genome duplication one copy of each gene was deleted. Similar relationships are seen between the *K. lactis* gene cluster *HHT1-TRP1-IPPI* and block 3; between *K. lactis*

YNL217W-RAP1-GYP7 and block 20; and between *K. marxianus* *RPL25-YNL305C* and block 49 (refs 17–19). In each of these cases the transcriptional orientation of all genes has been conserved between the two species. These results could be explained either by our model of degenerate tetraploidy or by multiple independent regional duplications, but in the latter case all four duplications must have occurred in *Saccharomyces* after the speciation.

For most of the 376 gene pairs in *S. cerevisiae*, the function of both genes is not known. Only a few pairs have functions that have clearly diverged, notably the genes encoding mitochondrial and peroxisomal isozymes of citrate synthase (*CIT1/CIT2*)⁶, the *RAS1/RAS2* genes²⁰, the genes *ACE2/SWI5* which encode transcription factors²¹, *TOR1/TOR2* which encode phosphatidylinositol kinases²², and *MYO2/MYO4* which encode myosins²³. The differences between other pairs seem less important, for example the myosin genes *MYO3/MYO5*, and several pairs of genes that encode cyclins (including *CLN1/CLN2*, *CLB1/CLB2* and *CLB5/CLB6*), nucleoporins (*NUP100/NUP116* and *NUP157/NUP170*), the $\alpha 1$ and $\alpha 2$ mating pheromones, and 18 pairs of protein kinases. Nevertheless, according to our model these genes have been retained in duplicate for about 10^8 years. Before the genome duplication their separate functions must either have been embodied in a single protein², or one of the functions did not exist (or, less likely, one of the functions was performed by a different gene that was supplanted). This implies that the physiology of the ancestral yeast may have been quite different from that seen today, and was perhaps more similar to that of *Kluyveromyces*. The most striking physiological difference between *Saccharomyces* and other yeasts is its ability to ferment sugars vigorously under anaerobic conditions, producing ethanol. The proposed genome duplication may have been instrumental in its evolutionary adaptation to anaerobic growth; for example, the duplicate genes include several pairs that are regulated differently under aerobic and anaerobic conditions (*CYC1* and *CYC7*; *COX5A* and *COX5B*), as well as several genes encoding sugar transporters. It may not be a coincidence that the estimated date of the genome duplication corresponds to the time when angiosperms (and their fruit) became abundant in the earth's flora²⁴. □

Methods

Data. Yeast proteome lists from the Yeast Protein Database (YPD), the *Saccharomyces* Genome Database (SGD) and Martinsried Institute for Protein

Figure 3 Phylogenetic analysis of *S. cerevisiae* gene pairs and their *K. lactis* or *K. marxianus* homologues. Bootstrap support for the indicated branching order, and estimated age of the *S. cerevisiae* duplication relative to the *Saccharomyces/Kluyveromyces* speciation, are shown. Sch., *Schizosaccharomyces*.

Sequences (MIPS) databases were reconciled. Ty elements and dubious open reading frames (ORFs) were excluded. The data set (5,790 proteins) and search results can be viewed at the URL <http://acer.gen.tcd.ie/~khwolfe/yeast>. Repetitive regions within proteins were masked using the SEG filter in BLAST. **Statistical analysis.** Chi-square tests (data not shown) indicate that duplicated genes in yeast are distributed in a highly non-random manner with regard to both the order in which homologous genes occur on pairs of chromosomes and the transcriptional orientations of those genes. A simultaneous origin of duplicate regions, as opposed to 55 independent duplications, is supported by a chi-square test on block orientations and by the lack of triplicated regions. The Poisson expectation if blocks were duplicated sequentially is for approximately 40 duplicated blocks, and 7 blocks that are replicated more than once (mainly triplicated). There is only one possible candidate for a triplicated region: the genes *YDR474C*, *YDR492W* and *GNP1* on chromosome IV and *YOR019W*, *YOL002C* and *SCM2* on chromosome XV meet our criteria for a duplicated chromosomal region; this is not shown in Fig. 2 because this area of chromosome IV overlaps with blocks 18 and 9, which have a higher density of homologues than the proposed chromosome IV/XV block. The three-gene match between chromosomes IV and XV is probably spurious, but even if this is counted as a triplication the departure from Poisson expectations is significant ($P = 0.001$).

Phylogenetic analysis. Protein sequences were aligned using default settings in ClustalW with manual editing to remove regions whose alignment was not clear. Branch lengths were estimated with correction for multiple hits²⁵. The mean age of duplication was estimated as $(B/(A+B) + C/(A+C))/2$, where A, B and C correspond to the lengths of branches A, B and C shown in Fig. 3. Confidence intervals were estimated by bootstrap analyses for genes where there were >10 inferred substitutions on branch A. One gene pair, *ORC1/SIR3*, was omitted because one of the yeast genes appeared more similar to its human homologue than to its duplicate.

Received 17 December 1996; accepted 20 April 1997.

- Ohno, S. *Evolution by Gene Duplication* (George Allen and Unwin, London, 1970).
- Hughes, A. L. The evolution of functionally novel proteins after gene duplication. *Proc. R. Soc. Lond. B* **256**, 119–124 (1994).
- Kaback, D. B. Yeast genome structure. In *The Yeasts* Vol. 6 (eds Wheals, A. E., Rose, A. H. & Harrison, J. S.), 179–222 (Academic, London, 1995).
- Olson, M. V. in *The Molecular and Cellular Biology of the Yeast Saccharomyces* Vol. 1 (eds Broach, J. R., Pringle, J. R. & Jones, E. W.) 1–40 (Cold Spring Harbor Laboratory Press, NY, 1991).
- Smith, M. M. Molecular evolution of the *Saccharomyces cerevisiae* histone gene loci. *J. Mol. Evol.* **24**, 252–259 (1987).
- Lalo, D., Stettler, S., Mariotte, S., Slonimski, P. P. & Thuriaux, P. Une duplication fossile entre les régions centromériques de deux chromosomes chez la levure. *C.R. Acad. Sci.* **316**, 367–373 (1993).
- Melnick, L. & Sherman, E. The gene clusters ARC and COR on chromosomes 5 and 10, respectively, of *Saccharomyces cerevisiae* share a common ancestry. *J. Mol. Biol.* **233**, 372–388 (1993).
- Goffeau, A. et al. Life with 6000 genes. *Science* **274**, 546–567 (1996).
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
- Ahn, S. & Tanksley, S. D. Comparative linkage maps of the rice and maize genomes. *Proc. Natl Acad. Sci. USA* **90**, 7980–7984 (1993).
- Spring, J. Vertebrate evolution by interspecific hybridisation—are we polyploid? *FEBS Lett.* **400**, 2–8 (1997).
- Roman, H. & Sands, S. M. Heterogeneity of clones of *Saccharomyces* derived from haploid ascospores. *Proc. Natl Acad. Sci. USA* **39**, 171–179 (1993).
- Kielland-Brandt, M. C., Nilsson-Tillgren, T., Gjermansen, C., Holmberg, S. & Pedersen, M. B. Genetics of brewing yeasts. In *The Yeasts* Vol. 6 (eds Wheals, A. E., Rose, A. H. & Harrison, J. S.) 223–254 (Academic, London, 1995).
- Ryu, S.-L., Murooka, Y. & Kaneko, Y. Genomic reorganization between two sibling yeast species, *Saccharomyces bayanus* and *Saccharomyces cerevisiae*. *Yeast* **12**, 757–764 (1996).
- Hendriks, L. et al. Phylogenetic relationships among ascomycetes and ascomycete-like yeasts as deduced from small subunit ribosomal subunit RNA sequences. *Syst. Appl. Microbiol.* **15**, 98–104 (1992).
- Heus, J. J., Zonneveld, B. J. M., Steensma, H. Y. & van den Berg, J. A. The consensus sequence of *Kluyveromyces fragilis* centromeres shows homology to functional centromeric DNA from *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **236**, 355–362 (1993).
- Stark, M. J. R. & Milner, J. S. Cloning and analysis of the *Kluyveromyces fragilis* *TRP1* gene: a chromosomal locus flanked by genes encoding inorganic pyrophosphatase and histone H3. *Yeast* **5**, 35–50 (1989).
- Larson, G. P., Castanotto, D., Rossi, J. J. & Malafa, M. P. Isolation and functional analysis of a *Kluyveromyces fragilis* *RAP1* homologue. *Gene* **150**, 35–41 (1994).
- Bergkamp-Steffens, G. K., Hoekstra, R. & Planta, R. J. Structural and putative regulatory sequences of *Kluyveromyces fragilis* ribosomal protein genes. *Yeast* **8**, 903–922 (1992).
- Hurwitz, N., Segal, M., Marbach, I. & Levitzki, A. Differential activation of yeast adenyl cyclase by Ras1 and Ras2 depends on the conserved N terminus. *Proc. Natl Acad. Sci. USA* **92**, 11009–11013 (1995).
- Dohrmann, P. R. et al. Parallel pathways of gene regulation: homologous regulators SWI5 and ACE2 differentially control transcription of *HO* and *chitinase*. *Genes Dev.* **6**, 93–104 (1992).
- Schmidt, A., Kunz, J. & Hall, M. N. TOR2 is required for organization of the actin cytoskeleton in yeast. *Proc. Natl Acad. Sci. USA* **93**, 13780–13785 (1996).
- Jansen, R. P., Dowzer, C., Michaelis, C., Galova, M. & Nasmyth, K. Mother cell-specific *HO* expression

in budding yeast depends on the unconventional myosin Myo4p and other cytoplasmic proteins. *Cell* **84**, 687–697 (1996).

- Friis, E. M., Chaloner, W. G. & Crane, P. R. (eds) *The Origins of Angiosperms and their Biological Consequences* (Cambridge Univ. Press, 1987).
- Fitch, W. M. & Margoliash, E. Construction of phylogenetic trees. *Science* **155**, 279–284 (1967).

Acknowledgements. We thank our colleagues in the yeast genome project; J. I. Garrels for use of the YPD database, which was central to this study; G. Butler, A. T. Lloyd, L. Skrabanek, C. Seioighe, B. Baum and R. Rothstein for comments; and S. Kossida, M. Lewis and R. Keogh for initial work on this project. Yeast genome sequencing in our laboratory was supported by the European Union. *In silico* analysis is supported by the European Union and Forbairt (to K.H.W.) and the Wellcome Trust (to D.C.S.).

Correspondence and requests for materials should be addressed to K.H.W. (e-mail: khwolfe@tcd.ie). Additional details of the dataset and results are available on the World-Wide Web at URL <http://acer.gen.tcd.ie/~khwolfe/yeast>.

A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells

Gosse J. Adema*, Franca Hartgers*, Riet Verstraten*, Edwin de Vries*, Gill Marland*, Satish Menon†, Jessica Foster†, Yuming Xu†, Pete Nooyen‡, Terrill McClanahan†, Kevin B. Bacon† & Carl G. Figdor*

* Departments of Tumour Immunology and ‡ Pathology, University Hospital Nijmegen St Radboud, Philips van Leydenlaan 25, 6525 EX Nijmegen, The Netherlands

† Departments of Immunology and Molecular Biology, DNAX Research Institute, Palo Alto, California 94304, USA

Dendritic cells form a system of highly efficient antigen-presenting cells. After capturing antigen in the periphery, they migrate to lymphoid organs where they present the antigen to T cells^{1,2}. Their seemingly unique ability to interact with and sensitize naive T cells gives dendritic cells a central role in the initiation of immune responses and allows them to be used in therapeutic strategies against cancer, viral infection and other diseases. How they interact preferentially with naive rather than activated T lymphocytes is still poorly understood. Chemokines direct the transport of white blood cells in immune surveillance^{3,4}. Here we report the identification and characterization of a C-C chemokine (DC-CK1) that is specifically expressed by human dendritic cells at high levels. Tissue distribution analysis demonstrates that dendritic cells present in germinal centres and T-cell areas of secondary lymphoid organs express this chemokine. We show that DC-CK1, in contrast to RANTES, MIP-1 α and interleukin-8, preferentially attracts naive T cells (CD45RA⁺). The specific expression of DC-CK1 by dendritic cells at the site of initiation of an immune response, combined with its chemotactic activity for naive T cells, suggests that DC-CK1 has an important role in the induction of immune responses.

Dendritic cells are key regulators in immune responses, capable of priming naive T cells. Their potent antigen-presenting capacity can be explained in part by their unique life cycle and their high expression of major histocompatibility complex (MHC) class I and II molecules as well as co-stimulatory molecules¹. Detailed molecular analysis of dendritic cell function has been hampered, however, by the low numbers of dendritic cells present in blood mononuclear cells. The mechanism by which dendritic cells interact with or activate resting naive T cells to initiate an immune response is not fully understood. One possibility is that secreted cytokines or chemokines preferentially attract or activate naive rather than activated T cells. We generated sufficient numbers of dendritic cells *in vitro*^{5,6} to prepare a panel of dendritic-cell cDNA libraries, which allowed us to analyse dendritic cells at the molecular level.

While searching at the genetic level for molecules expressed by dendritic cells, we identified a cDNA clone encoding a C-C chemokine that is abundantly expressed. The deduced sequence predicts a protein of 89 amino acids, of which the amino-terminal 20 amino acids contain all the characteristics of a signal peptide (Fig. 1a). The mature protein comprises 69 amino acids and is 63%, 38% and 33% identical to the chemokines MIP-1 α , RANTES and MCP-2, respectively (Fig. 1b). The idea that this chemokine is expressed abundantly by dendritic cells is supported by the finding that of 350 dendritic cell cDNAs analysed, six encode this C-C chemokine. We termed it DC-CK1.

An RNA species of 1.1 kb was readily detected by northern blot analysis in both total and poly(A)⁺ RNA isolated from dendritic cells (Fig. 2a). No DC-CK1 expression could be detected in T cell-, B cell- or three different monocyte cell lines. Moreover, freshly isolated resting or phytohaemagglutinin (PHA)/interleukin (IL)-2

activated peripheral blood mononuclear cells (PBMCs), the resting or PHA/IL-2 activated non-adherent PBMC fraction (T, B and NK cells), and the adherent PBMC fraction (monocytes) activated with lipopolysaccharide (LPS) all failed to express this chemokine. Two endothelial cell lines, representatives of a non-leukocyte cell type known to be capable of expressing a variety of chemokines^{3,4}, did not express the chemokine either (data not shown). Because dendritic cells abundantly expressing DC-CK1 were derived from purified monocytes cultured in the presence of GM-CSF and IL-4 (refs 5–7), we tested the ability of different stimuli to induce its expression in monocytes. Fetal calf serum (FCS), interferon (IFN)- γ and LPS were all unable to induce DC-CK1 mRNA (Fig. 2b). To demonstrate further the specific induction of this chemokine by GM-CSF plus IL-4 in monocytes, we cultured purified T cells in the presence of GM-CSF plus IL-4, with or without PHA/IL-2. No DC-CK1 mRNA could be detected in T cells under either of these

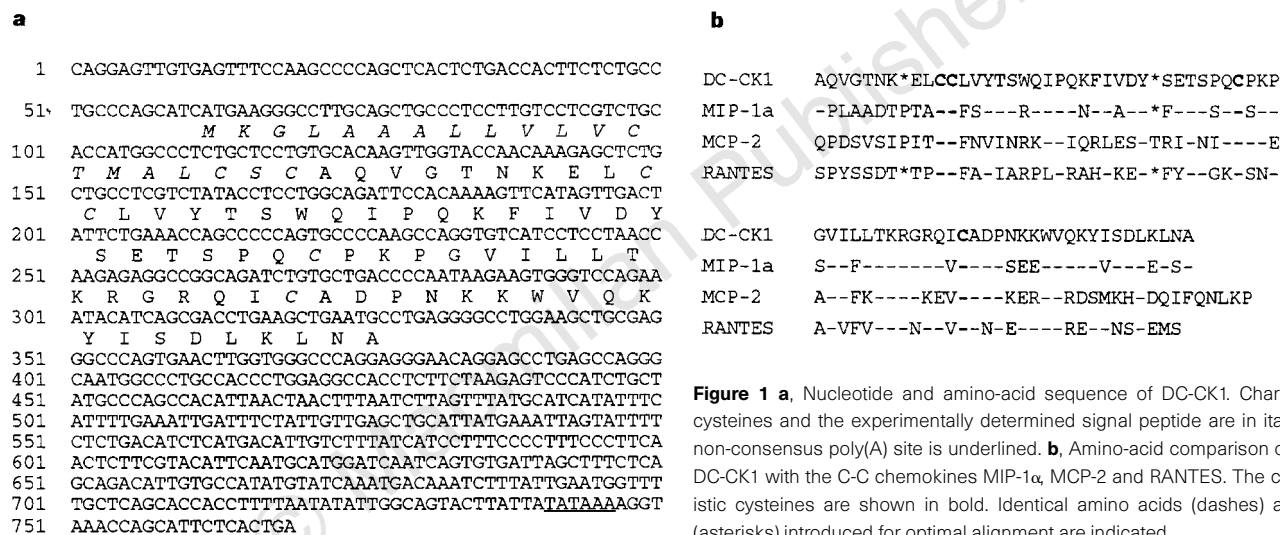


Figure 1 a, Nucleotide and amino-acid sequence of DC-CK1. Characteristic cysteines and the experimentally determined signal peptide are in italics. The non-consensus poly(A) site is underlined. **b**, Amino-acid comparison of mature DC-CK1 with the C-C chemokines MIP-1 α , MCP-2 and RANTES. The characteristic cysteines are shown in bold. Identical amino acids (dashes) and gaps (asterisks) introduced for optimal alignment are indicated.

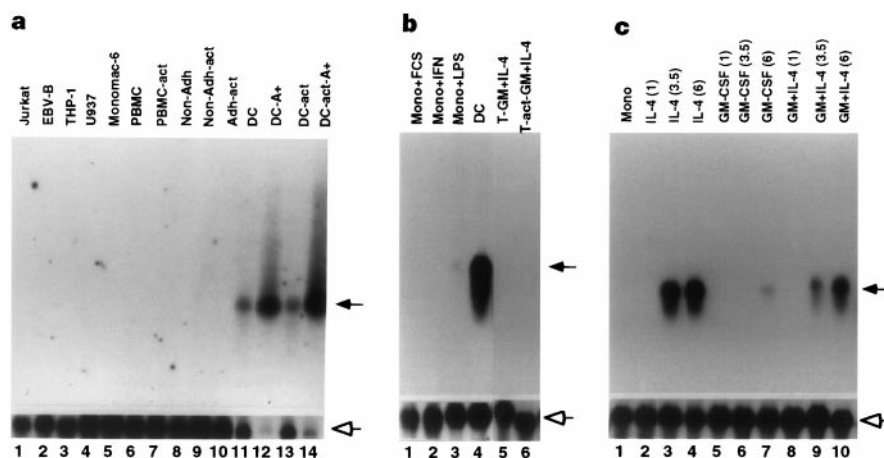


Figure 2 DC-CK1 mRNA is specifically expressed by dendritic cells. **a**, Northern blot analysis of DC-CK1 expression (filled arrow) in T (Jurkat), B (EBV-B) and monocyte *THP-1, U937, MonoMac-6) cell lines, freshly isolated resting or activated (act) PBMC subsets and GM-CSF plus IL-4-generated dendritic cells before (DC and D-A⁺ for polyA⁺ RNA) and after activation with TNF- α plus IL-1 α (DC-act and DC-act-A⁺ for polyA⁺ RNA) as indicated above each lane. **b**, Northern analysis of DC-CK1 expression in monocyte-derived dendritic cells and freshly isolated monocytes (mono) stimulated as indicated and T cells cultured in GM-

CSF plus IL-4 with or without activation with PHA/IL-2. **c**, Time course of DC-CK1 mRNA expression in freshly isolated monocytes cultured in the presence of IL-4, GM-CSF or the combination for the time (days) indicated in brackets. Northern blots were hybridized to a DC-CK1-specific and a control 28S ribosomal (open arrow) probe. Analogous results were obtained when cDNA libraries prepared from most of the same cell types were examined on Southern blots after digestion to release their inserts (not shown).

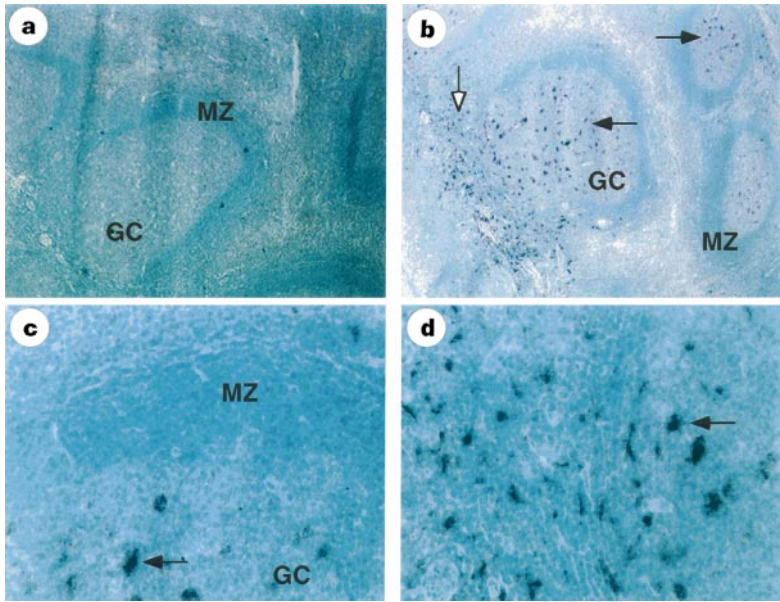


Figure 3 Detection of DC-CK1 in germinal centres and T-cell areas of a tonsil by *in situ* hybridization. Tonsil sections were stained with a sense (a) or antisense (b-d) DIG-labelled DC-CK1 RNA probe. Magnifications are $\times 40$ (a, b) and $\times 200$ for c (germal centre) and d (T-cell area). The filled arrow indicates staining in the

germinal centres (GC) and the open arrow in a T-cell area. No staining was observed in the mantle zone (MZ). An incubation time of 2-3 h with NBT/BCIP was sufficient to detect DC-CK1 mRNA, which is consistent with the abundance of DC-CK1 in the cDNA libraries.

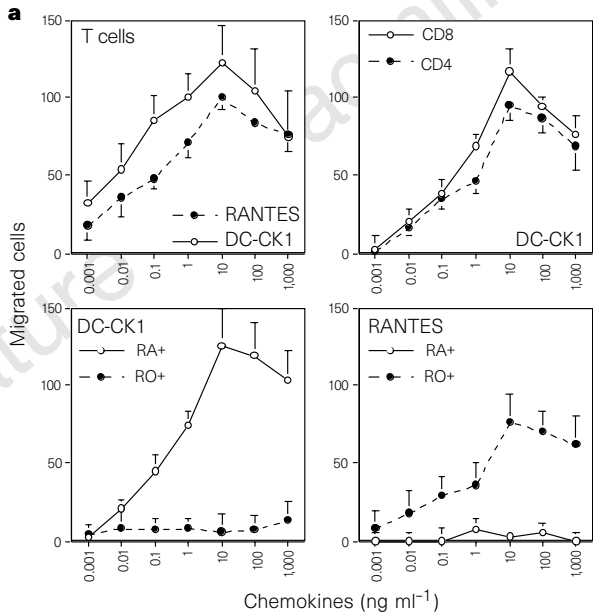
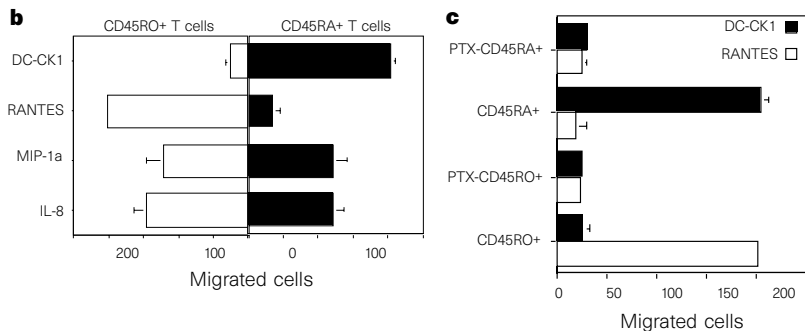


Figure 4 DC-CK1 is a potent chemoattractant for the CD45RA⁺ T-cell subset. **a**, Migration of purified T cells and the CD4⁺, CD8⁺, CD45RA⁺ and CD45RO⁺ T-cell subsets in response to DC-CK1 and RANTES is indicated as the number of migrated cells versus the amount of chemokine. Experiments using filters of 5- μ m and 8- μ m pore-size yielded similar results. Results shown are representative from experiments using 5- μ m pore-size filters. Cells on the filter are counted from 5 fields under high power. Each point represents mean ($\pm$ s.e.m.) number of migrating cells minus the number of cells in the medium control from 3 experiments performed in duplicate. **b**, **c**, Migration of CD45RA⁺ and CD45RO⁺ T cells in response to DC-CK1, RANTES, MIP-1 α and IL-8, and their sensitivity to 100 ng ml⁻¹ pertussis toxin. The concentration of chemokine yielding the optimal response (10 ng ml⁻¹ for all chemokines) is shown. Results are representative from experiments using 8- μ m pore-size filters. Each point represents mean ($\pm$ s.e.m.) number of migrating cells minus the number of cells in the medium control from 3 experiments performed in duplicate.



conditions (Fig. 2b). Splenocytes activated with IL-4 and anti-CD40 monoclonal antibodies also do not express DC-CK1 (data not shown). Taken together, these data demonstrate that DC-CK1 is primarily expressed in dendritic cells but not in any of the other leukocytes tested.

To investigate the time required for the expression of DC-CK1 and the dependence on GM-CSF and IL-4, a time-course experiment was performed using either GM-CSF, IL-4 or a combination of both. No DC-CK1 mRNA could be detected in freshly isolated monocytes, or monocytes cultured for 24 h in any cytokine combination (Fig. 2c). However, after 3.5 days of culture, DC-CK1 mRNA is abundantly present in cells cultured with either GM-CSF plus IL-4 or IL-4 alone, but not with GM-CSF alone. After 6 days, DC-CK1 mRNA is still present in abundance in GM-CSF plus IL-4 and IL-4 cultured cells, whereas only a small amount can be discerned with GM-CSF alone. This latter finding might be explained by the survival of contaminating dendritic cells residing in the monocyte fraction. Collectively, these data indicate that high-level expression of DC-CK1 is dependent on the presence of IL-4. The finding that prolonged incubation in the presence of IL-4 (3.5 days) is required for the induction of DC-CK1 mRNA is consistent with IL-4 being a key cytokine in directing the differentiation of monocytes towards the dendritic cell lineage, whereas GM-CSF is merely thought to act as a survival factor^{5,6}.

To determine the number of dendritic cells in our preparations that express DC-CK1 mRNA, we did *in situ* hybridization (ISH) on cytopins of dendritic cell cultures. The results demonstrated that most cells (65–90%) expressed DC-CK1 mRNA, excluding the possibility of a contaminating cell population being responsible for its production (data not shown). To investigate the expression of DC-CK1 *in situ*, ISH was performed on secondary lymphoid tissues, which are known to contain dendritic cells. DC-CK1-expressing cells were readily observed in T-cell areas (identified by CD34 staining of high endothelial venules⁸) and germinal centres of a tonsil (Fig. 3), consistent with the distribution and morphology of dendritic cells^{1,9}. A similar expression pattern of DC-CK1 mRNA was observed in lymph nodes. Staining of serial sections for DC-CK1 by ISH and with monoclonal antibodies directed against CD68 and DRC-1/CD21 demonstrated that the DC-CK1-producing cells in the germinal centres were distinct from tingible body macrophages and follicular dendritic cells, respectively (data not shown). As determined with anti-CD3, -CD19 and -CD14 monoclonal antibodies, the DC-CK1-positive cells are also distinct from T cells, B cells and monocytes (data not shown). The DC-CK1-expressing dendritic cells in germinal centres are likely to represent the recently identified T-cell stimulatory germinal-centre dendritic cells⁷.

Because dendritic cells are so important for the activation of unprimed T cells, we investigated the ability of DC-CK1 to function as a chemoattractant for different T-cell subsets. Freshly isolated T cells respond optimally to 10 ng ml⁻¹ DC-CK1 (Fig. 4c), which is similar to the amount required for the potent T-cell chemoattractant RANTES. CD4⁺ and CD8⁺ T cells respond equally to DC-CK1. In contrast, separation of T lymphocytes into resting (CD45RA⁺) or activated (CD45RO⁺) subpopulations demonstrated that the main T-cell population attracted by DC-CK1 consists of naive resting T cells. Checkerboard analysis demonstrated that the effect of DC-CK1 on resting T cells is chemotactic rather than chemokinetic in nature (data not shown). The T-cell chemoattractants RANTES, MIP-1 α and IL-8 do not show this specificity for naive CD45RA⁺ T cells^{10–12} (Fig. 4b). DC-CK1 may therefore be used by dendritic cells to preferentially attract naive T cells which, after recognition of peptide/MHC complexes presented by dendritic cells results in the induction of a primary immune response.

Chemokines induce chemotaxis when they interact with specific receptors that signal through heterotrimeric G proteins. As a consequence, chemotaxis is sensitive to pertussis toxin, a potent inhibitor of G α_i proteins¹³. Pretreatment of cells with pertussis toxin

completely abrogated the chemotactic response of CD45RA⁺ T cells to DC-CK1 (Fig. 4c). These data provide further evidence that DC-CK1 acts primarily on the CD45RA⁺ T-cell subset, and indicate that the response to DC-CK1 is a G protein-coupled receptor-mediated event.

The specific expression of a chemokine, DC-CK1, preferentially attracting naive T cells may be one of the mechanisms used by dendritic cells to interact preferentially with unprimed T cells, and is likely to be an important first step in the initiation of an immune response. Monoclonal antibodies directed against DC-CK1 will be useful in analysing the lineage relationship between subsets of dendritic cells, as well as in defining stages of their activation and maturation¹⁴. Further, such antibodies will be an important tool for detecting dendritic cells in different pathological conditions, including cancer and HIV infection. Notably, HIV-1 infection has recently been shown to be suppressed by chemokines and to be dependent on chemokine receptors as a cell entry cofactor^{15,16}. Further characterization of DC-CK1 and its receptor will make clear its function as an immunoregulator and its importance in the biology of dendritic cells, including their role and that of DC-CK1 in the pathogenesis of HIV. □

Methods

Cell culture. Dendritic cells were generated by culturing elutriated monocytes¹⁷ (>90% CD14⁺) in Iscove's medium with 5% FCS, 800 U ml⁻¹ GM-CSF (Schering-Plough, The Netherlands) and 500 U ml⁻¹ IL-4 (Schering-Plough) as described⁷. Dendritic were collected directly or after activation with LPS (2 μ g ml⁻¹), TNF- α (15 ng ml⁻¹) plus 75 LAF u ml⁻¹ IL-1 α (Hoffman LaRoche, Nutley, NJ) or 40% monocyte supernatant for 4 h or 16 h. A mixture of the 4-h and 16-h portions was used for northern analysis and cDNA library construction. The phenotype and effects of subsequent stimulation were confirmed by FACS analysis^{5–7}. For northern analysis, total PBMC and the non-adherent fraction of PBMC were activated with 1 μ g ml⁻¹ PHA and 20 U ml⁻¹ IL-2 (Cetus, Emeryville) (PBMC-act; Non-Adh act) for 3 days. The adherent PBMC fraction was activated with 2 μ g ml⁻¹ LPS for 2 days. Elutriated monocytes (>85% CD14⁺) were activated with 2 μ g ml⁻¹ LPS. 75 U ml⁻¹ IFN- γ (Boehringer Ingelheim, Alkmaar, The Netherlands) or 5% FCS for 4 days and elutriated T cells (>95% CD3⁺) with GM-CSF (800 U ml⁻¹) and IL-4 (500 U ml⁻¹) with or without 1 μ g ml⁻¹ PHA and 20 U ml⁻¹ IL-2 for 3 days. T-cells subsets used for chemotaxis were obtained by negative selection with the appropriate cocktails of monoclonal antibodies against CD14 (Leu-M3), CD19 (Leu-12), CD20 (Leu-16), CD56 (Leu-19), CD45RA (Leu-18), CD45RO (Leu-45RO) (all Becton Dickinson, Mountain View, USA), CD67 (IOM-67, Immunotech, Westbrook), glycophorin (10F7MN, ATCC Rockville), CD4 (OKM-1) and CD8 (BL12) from ficoll banded mononuclear cells using sheep anti-mouse M-450 Dynabeads (Dyna, Oslo, Norway). Purity was >94% as determined by FACS analysis.

cDNA library construction and northern blot analysis. Total RNA was isolated from the dendritic cell cultures described above using the guanidine thiocyanate/cesium chloride procedure. After isolation of poly(A)⁺ RNA (Oligotex, Qiagen), cDNA was synthesized and cloned into pSport1 using the superscript plasmid system (Gibco BRL). Nucleotide sequence analysis of 360 randomly picked clones was performed using the ABI system. For northern analysis, RNA isolated using the RNazol B method (Biotecx Lab., Houston, TX) was used. As a DC-CK1-specific probe, we used a random primed labelled 380-bp BSTX-I/NotI fragment comprising the 3' non-coding region. Spotblots containing MIP-1 α cDNA were included in each hybridization to ensure specificity. To control for the amount and quality of the RNA samples, blots were also hybridized with a 28S ribosomal probe¹⁸.

Recombinant chemokines and chemotaxis. The N terminus of DC-CK1 was defined by N-terminal sequencing of purified recombinant DC-CK1 produced in COS-7 cells using the pFLAG system (International Biotechnologies, New Haven, CT). To produce recombinant DC-CK1 used in the chemotaxis experiments, *Escherichia coli* strain X156F was transformed with the pOMP plasmid containing the mature DC-CK1 coding region. After lysis, the periplasmic fraction was purified on Q-sepharose and S-sepharose columns (Pharmacia) using a linear NaCl gradient (0–0.1 M). The DC-CK1-enriched

fractions were loaded on a reverse-phase column and eluted using a linear gradient of 2–80% acetonitrile. DC-CK1 protein concentration was estimated by densitometric scanning of a coomassie blue-stained gel containing lysozyme as a standard. RANTES, MIP-1 α and IL-8 were obtained from R&D Systems. T-cell migration was measured using 48-well chemotaxis chambers (Neuroprobe) as described¹⁹. In brief, chemokines in RPMI-1640 were added to the lower chamber and were separated from 10⁵ cells in RPMI-1640 with 10% FCS by either a 8- μ m or a 5 μ m PVP-free polycarbonate membrane (Poretics, Livermore). After incubation for 1 h, the membrane was removed and the upper side washed with PBS, scraped to remove residual cells and washed again. After methanol fixation and staining, the number of fully migrated cells was counted microscopically in 5 high-power fields ($\times$ 400) per well. Pertussis toxin (100 ng ml⁻¹) (Calbiochem) was used for 2 h. Each experiment was performed in duplicate, and experiments with DC-CK1, RANTES, MIP-1 α and IL-8 were performed in parallel in the same assay to make a direct comparison of their activities possible.

In situ hybridization. Cryosections (8 μ m) of tonsils and lymph nodes were fixed in 4% paraformaldehyde and pretreated with 2 μ g ml⁻¹ pepsin in 0.2 M HCl for 10 min and 0.1 M triethanol amine/0.25 acetic acid anhydride for 10 min. Sections were hybridized overnight with either a sense or an antisense DIG-labelled DC-CK1 RNA probe consisting of the 3' non-coding region generated by *in vitro* transcription (Boehringer Mannheim). Before incubation with anti-DIG-alkaline phosphatase monoclonal antibody the sections were treated with 40 U ml⁻¹ RNase I (Promega) to ensure specificity. After incubation for 2–3 h with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) the sections were stained with methylene green and embedded in Kaiser's. Immunostaining was performed as described²⁰.

Received 30 December 1996; accepted 24 April 1997.

- Steinman, R. M. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**, 271–296 (1991).
- Marland, G., Bakker, A. B. H., Adema, G. J. & Figdor, C. G. Dendritic cells in immune response induction. *Stem Cells* **14**, 501–507 (1997).
- Oppenheimer, J. J., Zachariae, C. O. C., Mukaida, N. & Matsushima, K. Properties of the novel proinflammatory supergene cytokine family. *Annu. Rev. Immunol.* **9**, 617–648 (1991).
- Schall, T. In *The Cytokine Handbook* (ed. Thompson, A.) 418–460 (Academic, New York, 1994).
- Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α . *J. Exp. Med.* **179**, 1109–1118 (1994).
- Romani, N. *et al.* Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180**, 83–93 (1994).
- Bakker, A. B. H. *et al.* Generation of anti-melanoma CTL from healthy donors after presentation of melanoma associated antigen derived epitopes by dendritic cells in vitro. *Cancer Res.* **55**, 5330–5334 (1995).
- Baumhueter, S. *et al.* Binding of L-selectin to the Vascular sialomucin CD34. *Science* **262**, 436–438 (1993).
- Grouard, G., Durand, I., Filgueira, L., Banchereau, J. & Liu, Y.-J. Dendritic cells capable of stimulating T cells in germinal centers. *Nature* **384**, 364–367 (1996).
- Schall, T. J., Bacon, K., Toy, K. J. & Goeddel, D. V. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* **347**, 669–672 (1991).
- Schall, T. J., Bacon, K., Camp, R. D. R., Kasper, J. W. & Goeddel, D. V. Human Mip-1 α and Mip-1 β chemokines attract distinct populations of lymphocytes. *J. Exp. Med.* **177**, 1821–1825 (1993).
- Ross, J. S., Mistry, K., Bacon, K. B. & Camp, R. D. Characterisation of the *in vitro* responsiveness of lymphocyte subsets to locomotor stimuli by immunocytochemical methods. *J. Immunol. Meth.* **140**, 219–225 (1991).
- Katz, A., Wu, D. & Simon, M. I. Subunits $\beta\gamma$ of heterotrimeric G protein activate $\gamma 2$ isoform of phospholipase C. *Nature* **360**, 686–689 (1992).
- Peters, J. H., Gieseler, R., Thiele, B. & Steinbach, F. Dendritic cells: from ontogenic orphans to myelomonocytic descendants. *Immunol. Today* **17**, 273–278 (1996).
- Cocchi, F. *et al.* Identification of RANTES, Mip-1 α and Mip-1 β as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* **270**, 1811–1815 (1995).
- Premack, B. A. & Schall, T. J. Chemokine receptors: Gateways to inflammation and infection. *Nature Med.* **11**, 1174–1178 (1996).
- Figdor, C. G., Bont, W. S., Touw, I., Roosnek, E. E. & de Vries, J. E. Isolation of functionally different human monocytes by counterflow centrifugation elutriation. *Blood* **60**, 46–53 (1982).
- Groningen, van J. J., Bloemers, H. P. & Swart, G. W. Identification of melanoma inhibitory activity and human melanoma cell lines with different metastatic capacity by messenger RNA differential display. *Cancer Res.* **15**, 6237–6243 (1995).
- Bacon, K. B., Camp, R. D., Cunningham, F. M. & Woollard, P. M. Contrasting *in vitro* lymphocyte chemotactic activity of the hydroxyl enantiomers of 12-hydroxy-5,8,10,14-eicosatetraenoic acid. *Br. J. Pharmacol.* **95**, 966–972 (1988).
- Mattijssen, V. *et al.* Clinical and immunopathological results of a phase II study of perilymphatically injected recombinant IL-2 in locally advanced head and neck squamous cell carcinoma. *J. Immunother.* **10**, 63–68 (1991).

Acknowledgements. We thank S. Zurawski, D. Gorman, F. Vega, R. Kastelein, M. Bell, K. Franz-Bacon, D. Figueroa, M. Koningswieser, R. Huijbens, C. Maass and L. Schalkwijk for assistance, and G. Zurawski, D. Ruiter and P. de Mulder for support. The DNAX Research Institute is supported by Schering Plough Corporation.

Correspondence and requests for materials should be addressed to G.J.A. (e-mail: g.adema@dent.kun.nl).

A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor

Robert D. Klein^{*,†}, Daniel Sherman^{*}, Wei-Hsien Ho^{*}, Donna Stone^{*}, Gregory L. Bennett[†], Barbara Moffat[‡], Richard Vandlen[‡], Laura Simmons[§], Qimin Gu[§], Jo-Anne Hong^{||}, Brigitte Devaux^{||}, Kris Poulsen^{*}, Mark Armanini^{*}, Chika Nozaki[†], Naoya Asai[†], Audrey Goddard[§], Heidi Phillips^{*}, Chris E. Henderson[#], Masahide Takahashi[†] & Arnon Rosenthal^{*}

Departments of ^{*}Neuroscience, [†]BioAnalytical Technology, [‡]Protein Biochemistry, [§]Molecular Biology and ^{||}Antibody Technology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

[¶]Department of Pathology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan

[#]INSERM U.382, Developmental Biology Institute of Marseille, 13288 Marseille 09, France

[§]Present address: Deltagen, Inc., 1031 Bing Street, San Carlos, California 94070, USA

Glial-cell-line-derived neurotrophic factor (GDNF) and neurturin (NTN) are two structurally related, potent survival factors for sympathetic, sensory and central nervous system neurons^{1–6}. GDNF mediates its actions through a multicomponent receptor system composed of a ligand-binding glycosyl-phosphatidylinositol (GPI)-linked protein (designated GDNFR- α) and the transmembrane protein tyrosine kinase Ret^{7–12}. In contrast, the mechanism by which the NTN signal is transmitted is not well understood. Here we describe the identification and tissue distribution of a GPI-linked protein (designated NTNR- α) that is structurally related to GDNFR- α . We further demonstrate that NTNR- α binds NTN ($K_d \sim 10$ pM) but not GDNF with high affinity; that GDNFR- α binds to GDNF but not NTN with high affinity; and that cellular responses to NTN require the presence of NTNR- α . Finally, we show that NTN, in the presence of NTNR- α , induces tyrosine-phosphorylation of Ret, and that NTN, NTNR- α and Ret form a physical complex on the cell surface. These findings identify Ret and NTNR- α as signalling and ligand-binding components, respectively, of a receptor for NTN and define a novel family of receptors for neurotrophic and differentiation factors composed of a shared transmembrane protein tyrosine kinase and a ligand-specific GPI-linked protein.

In searching for a neurturin receptor, we examined sequences deposited in public databases for similarity to the GDNF receptor α component (GDNFR- α)^{9,10}. Eight partial human cDNAs (Genbank accession numbers R02249, H12981, W73681, W73633, H05619, R02135, T03342 and HSC1KA111) were identified and found to encode parts of a single protein of 464 amino acids which we designated neurturin receptor α (NTNR- α). The human proteins hNTNR- α and hGDNFR- α display an overall 48% similarity, and the positions of their cysteine residues are conserved (Fig. 1). Both hNTNR- α and hGDNFR- α seem to be extracellular proteins that are attached to the outer cell membrane by means of a glycosyl-phosphatidyl inositol (GPI) modification. hNTNR- α has an amino-terminal signal peptide for secretion, three glycosylation sites, and a stretch of 17 carboxy-terminal hydrophobic amino acids preceded by a group of three small amino acids (Gly, Ser, Asn) defining a cleavage/binding site for GPI linkage (Fig. 1). Subsequently, a rat NTNR- α (rNTNR- α) was isolated and shown to be 94% identical to its human homologue (Fig. 1).

To examine whether NTNR- α could be a receptor for a neurotrophic factor such as NTN, the tissue distribution of NTNR- α mRNA was examined by *in situ* hybridization and compared with

that of GDNFR- α (Fig. 2 and data not shown). In the embryonic rat nervous system, mRNA for NTNR- α was found in the ventral midbrain where dopaminergic neurons are located, in parts of the ventral spinal cord where motor neurons are present and in the dorsal root ganglia (DRG) where sensory neurons reside. In addition, high levels of NTNR- α transcripts were found in developing sympathetic ganglia and peripheral nerves. Low levels of NTNR- α mRNA were detected in non-neuronal tissues, such as smooth and striated muscle of the embryonic gut, oesophagus, diaphragm and limb bud, which express high levels of GDNFR- α transcripts. In the adult rat brain, NTNR- α mRNA was detected in the substantia nigra, cortex, olfactory bulb and the dorsal horn of the spinal cord. Although NTNR- α mRNA and GDNFR- α mRNA were often coexpressed, the two transcripts occasionally displayed distinct tissue distribution. For instance, in the limb, GDNFR- α is expressed mainly in muscle cells but also around the brachial plexus, which innervates the muscle, whereas NTNR- α is found mainly in the brachial plexus. Similarly, in the embryonic bladder, NTNR- α is expressed in the muscle layer, whereas GDNFR- α is expressed in the underlying epithelia. Finally, in the gut, NTNR- α is expressed in the mucosal epithelium as well as in smooth muscle whereas GDNFR- α is expressed only in smooth muscle (Fig. 2). This pattern of expression is consistent with the hypothesis that NTNR- α mediates signals both inside and outside the nervous system, and suggests overlapping, yet distinct, biological roles for NTNR- α and GDNFR- α .

To determine whether NTNR- α could function as a receptor for NTN, binding experiments were performed. Equilibrium binding of human or mouse 125 I-NTN to recombinant soluble human (data not shown) or rat NTNR- α protein demonstrated that NTN binds specifically and reversibly to NTNR- α , and that the two proteins associate with an approximate K_d of 10 pM (Fig. 3). In contrast, neither human nor mouse NTN (at 1 nM) were able to displace 125 I-rGDNF from rGDNFR- α , and no binding of human or mouse 125 I-NTN to rGDNFR- α was detected. Taken together, these data are consistent with the idea that NTN interacts at high affinity solely with NTNR- α (Fig. 3). Although no high-affinity interaction between NTN and GDNFR- α was detected, we did observe, using higher concentrations of unlabelled NTN (10 nM), displacement of 125 I-rGDNF from GDNFR- α , suggesting that there is a low-affinity interaction between NTN and GDNFR- α ($K_d > 1$ nM) (data not shown).

To examine further whether NTNR- α is a specific receptor for NTN, competition binding experiments were performed using 125 I-rGDNF. Displaceable high-affinity binding of 125 I-rGDNF to recombinant soluble rGDNFR- α was readily observed ($K_d = 3$ pM) (Fig. 3), but no binding of 125 I-rGDNF (iodinated either on primary amino groups by using the Bolton-Hunter method, or on tyrosines by using the lactoperoxidase method) could be detected to either hNTNR- α or rNTNR- α . Similarly, no significant displacement of 125 I-labelled mouse NTN (125 I-mNTN) from NTNR- α was detected in the presence of unlabelled rGDNF (up to 1 nM). Thus GDNF seems to bind with high affinity to GDNFR- α but not to NTNR- α . As before, displacement of 125 I-mNTN from NTNR- α was observed when a high concentration (10 nM) of unlabelled GDNF was used, suggesting a low-affinity interaction between GDNF and NTNR- α ($K_d > 1$ nM) (data not shown). The specific high-affinity interactions between GDNF and GDNFR- α , and NTN and NTNR- α , were confirmed using competition binding to intact cells that express individual receptors (data not shown). Consistent with the prediction that NTNR- α is anchored to the cell surface by a GPI linkage, the binding of 125 I-NTN to cells expressing NTNR- α was significantly reduced by treatment with phosphoinositide-specific phospholipase C (PIPLC), an enzyme that specifically cleaves GPI linkages¹³ (Fig. 4a). Taken together, these findings support the idea that NTNR- α is a high-affinity, GPI-linked binding protein for NTN, and that GDNFR- α is a specific high-affinity binding protein for GDNF.

Consistent with the idea that NTNR- α participates in the response to NTN, we found that NTN can prevent the death of two populations of neurons that express NTNR- α : primary embryonic dopaminergic neurons (data not shown), and motor neurons (Fig. 4b). Moreover, in agreement with the finding that NTN and GDNF use distinct receptors, we detected differences in the efficacy (but not in the potency) of the two factors. Whereas GDNF at saturating concentrations promoted the survival of 100% of BDNF-responsive motor neurons, NTN prevented the death of only 50% of these cells (Fig. 4b).

To confirm that NTNR- α is a required mediator of the NTN signal, embryonic motor neurons were treated with PIPLC, and their survival in the presence of NTN or BDNF was monitored in culture. The capacity of saturating concentrations of NTN to support the survival of embryonic rat motor neurons was completely abolished by PIPLC treatment, whereas no decrease in the

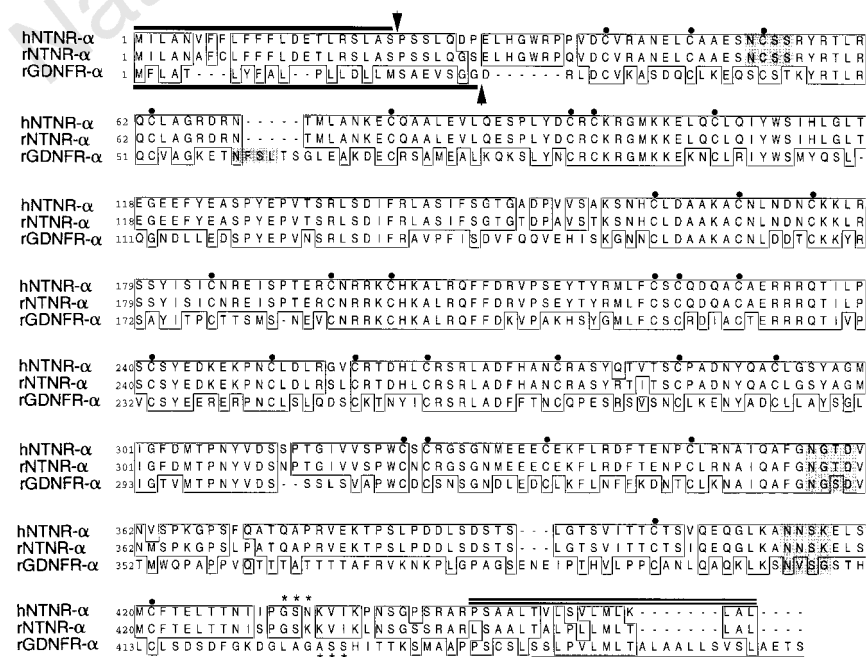


Figure 1 Primary structure of NTNR- α , and their homology to rat GDNFR- α . Signal peptides are indicated by solid lines, putative signal peptide cleavage sites are marked with arrows, potential glycosylation sites are shaded, the hydrophobic domains of the GPI attachment sites are doubly underlined, the small amino acid residues that constitute the cleavage/attachment site for GPI-linked proteins are marked with asterisks, and the consensus cysteine residues are indicated by filled circles.

response of motor neurons to brain-derived neurotrophic factor (BDNF) was caused by PIPLC. Moreover, when NTN was added to PIPLC-treated motor neurons in combination with soluble NTNR- α , the response to NTN (Fig. 4c), but not to GDNF (data not shown), was restored. Thus NTNR- α is a necessary and specific component of the NTN signalling cascade and has the properties expected of the ligand-binding subunit of a functional NTN receptor.

NTNR- α , like GDNFR- α , lacks a cytoplasmic domain and is anchored to the outer surface of the cell by GPI. Thus, transmission of the NTN signal to the interior of the cell must involve additional proteins. As the tyrosine-kinase receptor Ret, which by itself does not bind GDNF^{9,10} or NTN (data not shown), was found to be a signalling component of the GDNF receptor, we examined the possibility that it would also transduce the NTN signal following binding of NTN to NTNR- α . The human neuroblastoma TGW-I-nu cell line, which expresses endogenous *c-ret*^{14,15}, was exposed to NTN for 5 min, and the level of Ret tyrosine phosphorylation was determined. NTN induced phosphorylation of Ret (Fig. 4d), as well as two isoforms of the cytoplasmic kinase ERK (MAP kinase) (Fig. 4e) in this cell line. Furthermore, consistent with the idea that NTNR- α is a necessary mediator of the response to NTN, only residual tyrosine phosphorylation of Ret in response to NTN was observed in PIPLC-treated TGW-I-nu cells (Fig. 4d). The amount of tyrosine-phosphorylated Ret protein was greatly increased in PIPLC-treated TGW-I-nu cells when NTN was added together with a soluble NTNR- α (Fig. 4d).

As these findings suggested that Ret participates in the transmission of the NTN signal, we investigated whether it is part of a putative NTN receptor complex. TGW-I-nu cells were exposed to NTN and then lysed with a mild detergent. Protein complexes were

immunoprecipitated with a polyclonal antibody to Ret and then analysed on a western blot using a polyclonal antibody to NTN. Consistent with the idea that NTN and Ret interact physically on the cell surface, NTN was readily co-immunoprecipitated by Ret antibodies (Fig. 4f). To confirm that NTNR- α is also present in the NTN–Ret protein complex, human embryonic kidney 293 cells were transiently transfected with *c-ret* alone, with an epitope-tagged NTNR- α alone, or with a combination of expression vectors for *c-ret* and an epitope-tagged NTNR- α , and were exposed to NTN before being lysed with a mild detergent¹⁶. Protein complexes were then immunoprecipitated with polyclonal antibody to Ret and analysed on a western blot using a monoclonal antibody to the epitope-tagged NTNR- α . No protein complex could be detected in cells that were transfected with Ret alone or with NTNR- α alone, either in the presence or absence of NTN (Fig. 4g, and data not shown). In contrast, in cells that expressed both proteins, residual NTNR- α could be co-immunoprecipitated by Ret antibodies in the absence of NTN. Further, addition of NTN led to a significant increase in the amount of NTNR- α that could be co-immunoprecipitated by the Ret antibodies (Fig. 4g). These findings support the hypothesis that NTN, NTNR- α and Ret form a complex on the cell surface; that Ret and NTNR- α are components of a functional NTN receptor; and that NTNR- α is an important intermediary in the interaction between NTN and Ret.

Taken together, our results define a candidate receptor for NTN and demonstrate the existence of a family of multicomponent receptors composed of a shared signalling subunit, the transmembrane tyrosine kinase receptor Ret, and receptor-specific ligand-binding subunits that are GPI-linked (Fig. 5). The findings are consistent with the observation that Ret-deficient mice display more severe deficits in the superior cervical ganglion sympathetic

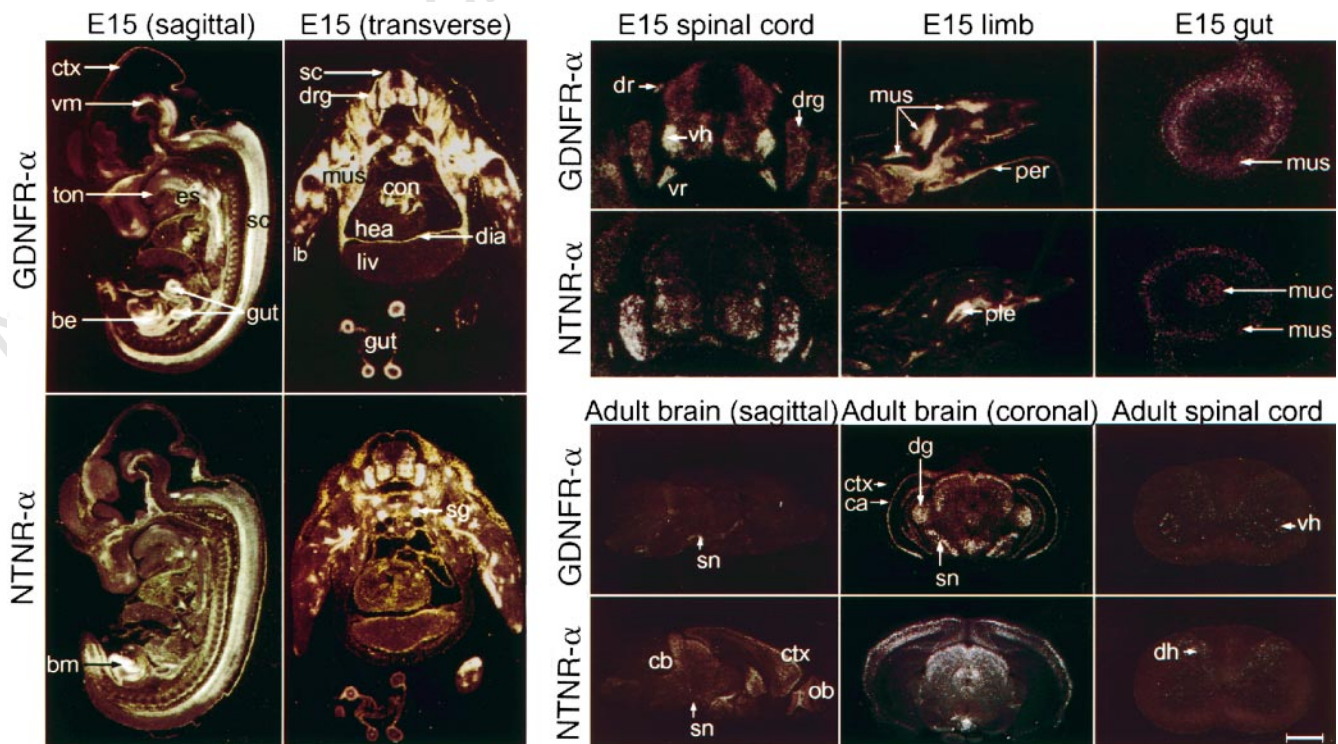


Figure 2 Tissue distribution of mRNA for NTNR- α and GDNFR- α . Abbreviations: vm, ventral midbrain; es, oesophagus; ctx, cortex; sc, spinal cord; ton, tongue; be, bladder epithelium; sg, sympathetic ganglia; bm, bladder muscles; dia, diaphragm; mus, muscle; con, cardiac conductive system; sn, substantia nigra; dg, dentate gyrus; ob, olfactory bulb; cb, cerebellum; ca, cornu ammonis; dr, dorsal root; drg, dorsal root ganglia; vr, ventral root; vh, ventral horn; muc, mucosal

epithelial; pte, brachial plexus nerves; liv, liver; hea, heart. Scale bar, 1.1 mm in E15 sagittal section; 800 μ m in E15 transverse section; 240 μ m in E15 spinal cord; 590 μ m in E15 limb; 93 μ m in E15 gut; 3.6 mm in adult brain (sagittal); 2.4 mm in adult brain (coronal); 900 μ m in adult spinal cord. The E15 transverse and spinal cord sections were exposed for different times.

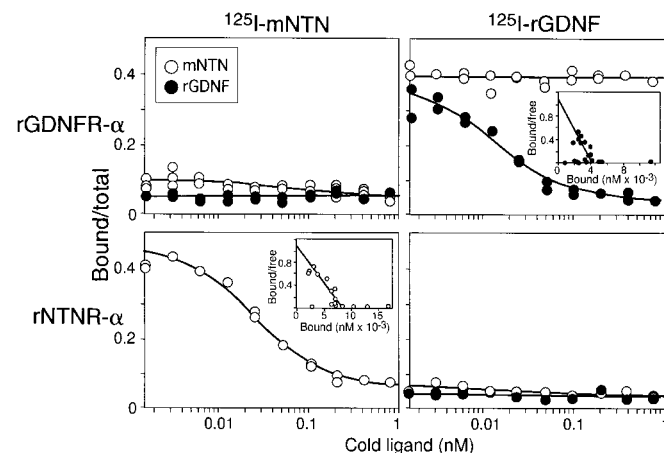


Figure 3 Binding of iodinated NTN and GDNF to NTNR- α and GDNFR- α . Binding of 125 I mouse NTN (125 I-mNTN) (5 pM) or 125 I rat GDNF (125 I-rGDNF) (5 pM) to rat NTNR- α (rNTNR- α) or rat GDNFR- α (rGDNFR- α). Human NTNR- α displayed a similar binding specificity to rat NTNR- α (data not shown). As depicted by the Scatchard analysis (insets), GDNF binds GDNFR- α with an approximate K_d value of 3 pM (similar K_d values were reported in a cell-based assay¹⁰), and NTN binds NTNR- α with an approximate K_d value of 10 pM.

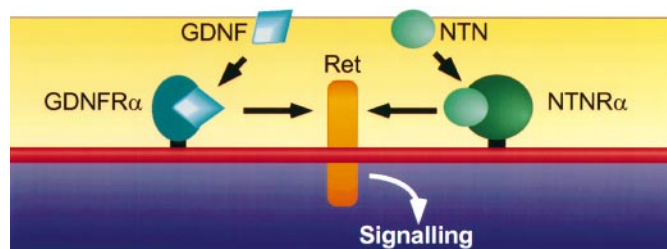


Figure 5 A schematic representation of activation of Ret by NTN or GDNF. NTN and GDNF each bind to a unique GPI-linked ligand-binding protein. This ligand-binding protein complex can bind to and activate a shared transmembrane tyrosine kinase receptor, Ret.

neurons^{17,18} than GDNF-deficient mice^{19–21} and suggest that the distinct biological activities of the GDNF and NTN may be determined by the different tissue distributions of their respective ligand-binding receptor components, rather than by their ability to activate different signalling systems.

Our findings provide a biological rationale for the evolution of what appeared to be a needlessly complex way to activate a tyrosine kinase²². With the discovery that Ret is shared by GDNF and NTN, it is now apparent that changing the ligand-binding molecule allows recruitment of the same signalling system by multiple growth factors. Similar 'cost-effective' strategies to make use of a single transmembrane signalling system by multiple growth factors appear to be used by cytokines^{16,23}, members of the transforming growth factor protein family²⁴, and bacterial endotoxins^{25,26}.

The discovery of this receptor system further defines a new model of signal transduction and highlights the diverse strategies that are used to transmit extracellular signals in the vertebrate nervous system. □

Methods

NTNR- α cloning and expression constructs. The partial cDNA sequence encoded by the expressed sequence tag (EST) cDNA clones was extended using 5' and 3' Marathon RACE reactions (Clontech) on human spleen mRNA, using conditions supplied by the manufacturer. Additional cDNA clones for NTNR- α were identified by screening a human fetal brain cDNA library (Stratagene)

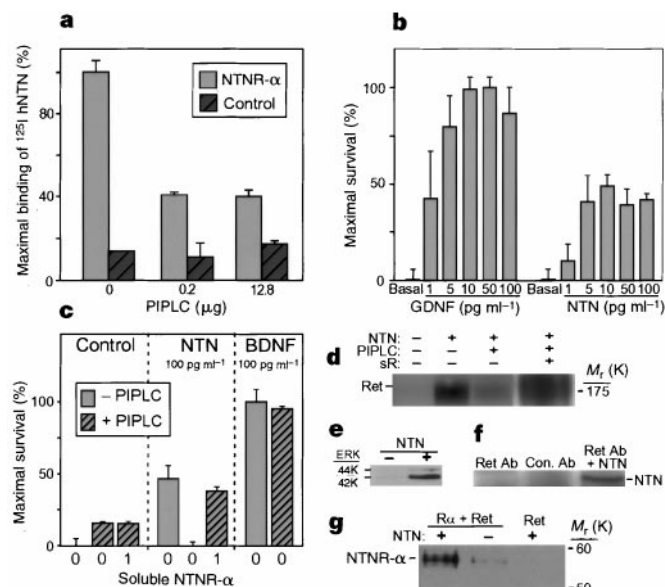


Figure 4 Interaction between NTN, NTNR- α and Ret. **a**, Binding of 125 I-NTN to cells expressing NTNR- α was reduced by 60% after treatment with PIPLC. **b**, Survival response of embryonic rat spinal motor neurons to GDNF or NTN, expressed as a percentage of the maximal survival in the presence of GDNF. In agreement with its receptor distribution, NTN is a potent survival factor for spinal motor neurons. **c**, Survival response of embryonic rat spinal motor neurons to NTN or BDNF in the presence of PIPLC and soluble NTNR- α . PIPLC treatment completely abolished the survival response to NTN without changing the response to BDNF. Soluble NTNR- α restored the response of PIPLC-treated motor neurons to NTN. **d**, NTN induces tyrosine phosphorylation of Ret in TGW-I-nu cells. Phosphorylation was significantly reduced in the presence of PIPLC and is restored in the presence of soluble NTNR- α (SR). **e**, NTN induces phosphorylation of ERK in TGW-I-nu cells. **f**, NTN and Ret form a complex in TGW-I-nu cells. **g**, Recombinant NTNR- α and Ret form a protein complex in 293 cells. The amount of this protein complex is significantly increased in the presence of NTN. Ret, cells transfected with Ret alone. R α + Ret, cells transfected with Ret and NTNR- α .

and a rat brain cDNA library (Clontech) using standard protocols. For mammalian protein expression, the complete open reading frame (ORF) was amplified using PCR and cloned into a CMV-based expression vector. The NTNR- α -IgG expression construct was made by cloning the first 432 amino acids of the receptor (which lacks a GPI linkage site) in front of the human Fc (IgG2a) sequence. For co-precipitation experiments, an epitope tag was inserted between the signal peptide and the mature coding sequence of NTNR- α . Soluble NTNR- α was produced as a C-terminal His-tagged protein in HEK 293 cells, purified by Ni-NTA chromatography²⁷, and tested for NTN binding. **In situ hybridization.** Rat embryos at embryonic day 15.5 (E15.5) were immersion-fixed overnight at 4°C in 4% paraformaldehyde, then cryoprotected overnight in 15% sucrose. Adult rat brains and spinal cords were frozen fresh. All tissues were sectioned at 16 μ m, and processed for *in situ* hybridization using 33 P-UTP labelled RNA probes³. Sense and antisense probes were derived for the N-terminal regions of NTNR- α or GDNFR- α using T7 polymerase.

Equilibrium binding analysis. For receptor-binding experiments, conditioned media from 293 cells transiently transfected with the NTNR- α -IgG or GDNFR- α -IgG constructs were incubated with approximately 5 pM 125 I-hNTN, 125 I-mNTN or 125 I-rGDNF, along with the appropriate cold ligand at concentrations between 0.8 nM and 1.56 pM (2-fold dilutions) in PBS containing 2 mg ml⁻¹ BSA (Sigma) and 0.05% Brij 96 (Fluka) for 4 h at room temperature (in the absence of cold ligands, 15–25% of the receptors were occupied). Protein A Sepharose CL-4B beads (~50 μ l; Pharmacia) were added to each reaction and the receptor–ligand complex was precipitated after

incubation for 1 h at room temperature. After washing with PBS containing 0.2 mg ml⁻¹ BSA, specific counts were measured. The IGOR program was used to determine K_d . Cell-based equilibrium binding analysis⁹ was used to confirm the specificity of GDNFR- α and NTNR- α for GDNF and NTN, respectively. Each binding experiment was repeated at least 4 times. For PIPLC analysis, 293 cells transiently transfected with either the full-length NTNR- α expression construct or an irrelevant plasmid were incubated with ~20,000 c.p.m. ¹²⁵I-hNTN in the presence of the indicated amounts of PIPLC for 30 min at room temperature. The cells were washed with ice-cold PBS containing 0.2 mg ml⁻¹ BSA, and cell-associated ¹²⁵I was measured.

Survival assays. E14 rat motor neurons were purified, plated and grown in duplicate wells in L15 medium with the N2 supplement and 2% horse serum²⁸. After 3 days in culture, only 30–40% of the motor neurons initially present survived in basal medium, whereas at saturating concentrations of GDNF or BDNF nearly all motor neurons remained alive. The increase in the number of surviving neurons in the presence of saturating concentrations of GDNF compared to basal medium was taken as 100% (maximal) survival; percentage of maximal survival under the indicated conditions is shown in Fig. 3b. PIPLC (2 μ g ml⁻¹) was added to the indicated samples 2 h before, as well as 12 h and 24 h after, addition of the indicated growth factors.

Tyrosine phosphorylation. To assay for tyrosine phosphorylation, cells were incubated for 1 h at 37°C with or without PIPLC and then exposed to NTN with or without soluble NTNR- α (10 μ g ml⁻¹) for 5–10 min at 37°C. Cells were then removed from the plates with 2 mM EDTA in PBS and lysed with ice-cold buffer (comprising, in mM 10 sodium phosphate (pH 7.0), 100 NaCl, 1% NP40, 5 EDTA, 100 sodium vanadate, 2 mM PMSE, and 0.2 units of aprotinin) and used for immunoprecipitation with antiserum raised against the 19 amino-acid C terminus of Ret, following by binding to protein A–Sepharose. The immunoprecipitated proteins were released by boiling in SDS sample buffer, separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with anti-phosphotyrosine antibody (Upstate Biotechnology). Detection was with an ECL western blotting detection system (Amersham Life Science).

Co-immunoprecipitation. To examine the formation of protein complexes, TGW-I-nu cell were exposed to 500 ng ml⁻¹ of NTN. Protein complexes were immunoprecipitated with Ret antibodies, transferred onto a nitrocellulose filter, and analysed with polyclonal antibody against NTN. Alternatively, HEK 293 cells were transiently transfected with expression vectors for Ret, an epitope-tagged NTNR- α , or a combination of an epitope-tagged NTNR- α and Ret. Cells were stimulated with NTN as indicated and lysed with brij 96 detergent (Fluka)¹⁶. Putative immune complexes were immunoprecipitated with a polyclonal antibody against Ret, transferred onto a nitrocellulose filter, and analysed with a monoclonal antibody against the epitope-tagged NTNR- α .

Received 7 March; accepted 1 May 1997.

- Lin, L. H., Doherty, D. H., Lile, J. D., Bektesh, S. & Collins, F. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130–1132 (1993).
- Kotzbauer, P. T. et al. Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* **384**, 467–470 (1996).
- Henderson, C. E. et al. GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266**, 1062–1064 (1994).
- Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A. & Davies, A. M. GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15**, 821–828 (1995).
- Arenas, E., Trupp, M., Akerud, P. & Ibáñez, C. F. GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. *Neuron* **15**, 1465–1473 (1995).
- Trupp, M. et al. Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J. Cell Biol.* **130**, 137–148 (1995).
- Takahashi, M., Ritz, J. & Cooper, G. M. Activation of a novel human transforming gene, *ret*, by DNA rearrangement. *Cell* **42**, 581–588 (1985).
- Takahashi, M. & Cooper, G. M. *ret* Transforming gene encodes a fusion protein homologous to tyrosine kinases. *Mol. Cell. Biol.* **7**, 1378–1385 (1987).
- Treanor, J. J. S. et al. Characterization of a multicomponent receptor for GDNF. *Nature* **382**, 80–83 (1996).
- Jing, S. et al. GDNF-induced activation of the *ret* protein tyrosine kinase is mediated by GDNFR- α , a novel receptor for GDNF. *Cell* **85**, 1113–1124 (1996).
- Trupp, M. et al. Functional receptor for GDNF encoded by the *c-ret* proto-oncogene. *Nature* **381**, 785–789 (1996).
- Durbec, P. et al. GDNF signalling through the Ret receptor tyrosine kinase. *Nature* **381**, 789–793 (1996).
- Kong, J. A., Yang, M., Henner, D. J., Volwerk, J. J. & Griffith, O. H. High-level expression in *Escherichia coli* and rapid purification of phosphatidylinositol-specific phospholipase C from *Bacillus cereus* and *Bacillus thuringiensis*. *Protein Expr. Purif.* **2**, 51–58 (1991).
- Ikeda, I. et al. Specific expression of the *ret* proto-oncogene in human neuroblastoma cell lines. *Oncogene* **5**, 1291–1296 (1990).
- Takahashi, M., Buma, Y. & Taniguchi, M. Identification of the *ret* proto-oncogene products in neuroblastoma and leukemia cells. *Oncogene* **6**, 297–301 (1991).
- Davis, S. et al. Released form of CNTF receptor α component as a soluble mediator of CNTF responses. *Science* **259**, 1736–1739 (1993).

- Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. & Pachnis, V. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367**, 380–383 (1996).
- Durbec, P. L., Larsson-Blomberg, L. B., Schuchardt, A., Costantini, F. & Pachnis, V. *Development* **122**, 349–358 (1996).
- Pichel, J. G. et al. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73–76 (1996).
- Sánchez, M. P. et al. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70–73 (1996).
- Moore, M. W. et al. Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76–79 (1996).
- Lindsay, R. M. & Yancopoulos, G. D. GDNF in a bind with known orphan: Accessory implicated in new twist. *Neuron* **17**, 571–574 (1996).
- Ip, N. Y. et al. CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69**, 1121–1132 (1992).
- Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. & Massague, J. Mechanism of activation of the TGF- β receptor. *Nature* **370**, 341–347 (1994).
- Lee, J.-D. et al. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. *Proc. Natl Acad. Sci. USA* **90**, 9930–9934 (1993).
- Pugin, J. et al. Lipopolysaccharide (LPS) activation of human endothelial and epithelial cells is mediated by LPS binding protein and soluble CD14. *Proc. Natl Acad. Sci. USA* **90**, 2744–2748 (1993).
- Hochuli, E., Dobeli, H. & Schacher, A. New metal chelate adsorbent selective for proteins and peptides containing neighboring histidine residues. *J. Chromatogr.* **411**, 177–184 (1987).
- Henderson, C. E. et al. in *Nerve Cell Culture: A Practical Approach* (eds Cohen, J. & Wilkin, G.) 69–81 (Oxford Univ. Press, 1995).

Acknowledgements. We thank J. Milbrandt and E. Johnson (who were supported by NIH grants) for providing unpublished information and reagents; W. Anstine for preparing the figures; E. Berry for help with the manuscript; A. Ryan for help with the *in situ* hybridization analysis; M. Vasser, P. Jhurani and P. Ng for synthetic oligonucleotides; M. Yang for the PIPLC enzyme; and V. Arce for help with motor neuron cultures. This work was supported in part by INSERM, Association Française contre les Myopathies (AFM) and Institut pour la Recherche sur la Moelle Epinière (IRME) to C.E.H., and by grants-in-aid from scientific research from the Ministry of Education, Science and Culture of Japan to M.T.

Correspondence and requests for materials should be addressed to A.R. (e-mail: ar@gene.com).

Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase

Anna Buj-Bello*[§], Jimi Adu*[§], Luzia G. P. Piñón*, Antony Horton*, Jane Thompson*, Arnon Rosenthal†, Miguel Chinchetru‡, Vladimir L. Buchman* & Alun M. Davies*

* School of Biological and Medical Sciences, Bute Medical Buildings, University of St Andrews, St Andrews, Fife KY16 9AT, UK

† Department of Neuroscience, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

‡ Present address: Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de León, 24007 León, Spain

§ These authors contributed equally to this work.

Neurturin (NTN)¹ is a recently identified homologue of glial-cell-line-derived neurotrophic factor (GDNF)². Both factors promote the survival of a variety of neurons^{1–5}, and GDNF is required for the development of the enteric nervous system and kidney^{6–8}. GDNF signals through a receptor complex consisting of the receptor tyrosine kinase Ret and a glycosyl-phosphatidylinositol (GPI)-linked receptor termed GDNFR- α ^{9–13}. Here we report the cloning of a new GPI-linked receptor termed NTNR- α that is homologous with GDNFR- α and is widely expressed in the nervous system and other tissues. By using microinjection to introduce expression plasmids into neurons, we show that coexpression of NTNR- α with Ret confers a survival response to neurturin but not GDNF, and that coexpression of GDNFR- α with Ret confers a survival response to GDNF but not neurturin. Our findings indicate that GDNF and neurturin promote neuronal survival by signalling through similar multicomponent receptors that consist of a common receptor tyrosine kinase and a member of a GPI-linked family of receptors that determines ligand specificity.

GDNFR- α	<u>MELALLYLALPLADVLL-S--AEVSGLPGGDRL-----D</u> <u>NKASDQCL</u>	40
NTNR- α	<u>MILANAFECIVLFVDET</u> <u>LRSLAAPP</u> <u>S-PPGQD-LQGW</u> <u>RV</u> <u>PVD</u> <u>Q</u> <u>IRANKLCA</u>	48
GDNFR- α	KEQSSTKYRTLROCVAGKESNFSRATGLEA-KDECKSAMEALKQKS-LY	88
NTNR- α	AEGSSSRYRTLROCLAGRDRN---TML-ANK-ECDAALEVL-QESPLY	91
GDNFR- α	NCRCKRGMKKEKNCLRIYWSMYQSL-QGNDLLEDSPYEPVNSRLSDIFRL	137
NTNR- α	DCRCKRGMKKEKICLLQVYWSIHLGLAEGEEFYEASPYEPITSRLSDIFRL	141
GDNFR- α	APIVSV-EPVL-SKGNCLDAAKANLNDTCRFRSAYITPISSTS-NE	184
NTNR- α	ASIFSGMDPATNSKSNHCLDAAKANLNDNCKRLRSGYISTCSKEISATE	191
GDNFR- α	ICNKRCKHKALRLFFDKVPPKHSYGMLEFCSCRDVACTERRRQTIVPVCSY	234
NTNR- α	HCRRCKHKALRQFFDNVPSEYTYRLLEFCCKDQACAEPRRQTIVPVCSY	241
GDNFR- α	EDREKPNCLNLOESCKKNYICRSRLADFFTNCOPESSRVSSCLKENYAD	284
NTNR- α	EDKEKPNCLDLRNVCRAHLCRSRLADFHANCOASFQSLTSCPGDNYQAC	291
GDNFR- α	LLAYSGLIGTVMTPNYIDSSSLSV--APWDCSNGNDIDECRKFLNFFQ	332
NTNR- α	LGSYTGLIGFDMTPNYVDASTTITISPWCSCKGSGNLEEECEKFLRDFT	341
GDNFR- α	DNTCLKNAIQAFNGTVDNVW--QPILPVQTTTATTTASRLKNTGSETT	380
NTNR- α	ENPCLRNAIQAFNGTVDNLSPKNPSPPITMLPKVEK-SPALPDDINDS	389
GDNFR- α	NNEIPTHNDSPACANLQAKKRKSNEVSVDTECLNENAIGKDNTPGVSTS	430
NTNR- α	NTMYDTSI-ITTTTSIQ-EHGQKLKSKESQSLCYSETQLTTDTPDQKTF	437
GDNFR- α	HISSENSFALPTS-FYPSTPLILMTIALSLFLFLSSSVVL	469
NTNR- α	VDQKAAGSRHRAARILPAVPIVLLKLLL	465

Figure 1 Aligned sequences of chicken GDNFR- α and NTNR- α . Identical amino acids are marked with asterisks and the conserved cysteines are enclosed in vertical boxes. The N-terminal, hydrophobic, putative signal peptides are underlined; the C-terminal hydrophobic domain is double-underlined; and the putative binding/cleavage consensus sequences for GPI linkage are enclosed in horizontal boxes. GenBank accession numbers: GDNFR- α , U90541; NTNR- α , U90542.

We have screened an embryonic-day-10 (E10) chicken brain cDNA library at low stringency using the full coding region of mouse GDNFR- α as a probe, and isolated two sets of overlapping cDNAs clones encoding chicken GDNFR- α and a GDNFR- α homologue designated, for reasons given below, neurturin receptor α (NTNR- α) (Fig. 1). The predicted chicken GDNFR- α is a protein of 469 amino acids which has 80% identity with rodent GDNFR- α . NTNR- α is a protein of 465 amino acids that has 49% identity with chicken GDNFR- α , including conservation of all 30 cysteines. Both GDNFR- α and NTNR- α possess an amino-terminal, hydrophobic, putative signal peptide for secretion, and the characteristic carboxy-terminal feature of GPI-linked proteins: a C-terminal hydrophobic domain separated by a hydrophilic linker region from a cleavage/binding consensus sequence for GPI linkage (Ser-Thr-Ser for GDNFR- α , and Ala-Gly-Ser for NTNR- α)¹⁴. These features suggest that both GDNFR- α and NTNR- α are secreted proteins attached to the extracellular surface of the plasma membrane.

To ascertain the potential involvement of NTNR- α in the survival responses of developing neurons to neurotrophic factors and to compare its function with that of GDNFR- α , we used microinjection to introduce expression plasmids encoding NTNR- α and GDNFR- α into cultured neurons that do not survive with either GDNF or neurturin. From a survey of several different populations of neurons, we found that sympathetic neurons of the superior cervical sympathetic ganglion (SCG) of postnatal day 4 (P4) mice are not supported by either neurturin or GDNF in culture. Reverse

transcription-polymerase chain reaction (RT-PCR) analysis showed that these neurons do not express endogenous messenger RNAs encoding NTNR- α , GDNFR- α or Ret, whereas mouse nodose neurons, which survive in response to GDNF and neurturin, express these mRNAs (Fig. 2a). Furthermore, because postnatal mouse SCG neurons die rapidly in defined medium lacking neurotrophic factors, they are ideal for examining the involvement of ectopically expressed receptors in mediating the responses of neurotrophic factors.

Ectopic expression of either NTNR- α or GDNFR- α alone in SCG neurons has a negligible effect on their survival (Fig. 2b) and does not confer a survival response to either GDNF or neurturin. This is consistent with the idea that GPI-linked receptors cannot mediate responses to their ligands without the appropriate transmembrane signalling proteins, such as Ret in the case of GDNFR- α ^{9,10} and gp130 and LIFR- β in the case of CNTFR- α ¹⁵.

Because Ret is an essential signalling component of the GDNF receptor complex⁹⁻¹³, we co-injected neurons with expression plasmids for Ret and either NTNR- α or GDNFR- α to see whether neurons expressing both of these receptor components would exhibit specific survival responses to GDNF and neurturin. Ectopic expression of Ret alone promoted the survival of approximately 10% of SCG neurons in the absence of added factors (Fig. 2b). However, neither GDNF nor neurturin significantly increased the survival of Ret-expressing neurons ($P > 0.05$, t -test, $n = 6$), suggesting that Ret alone is not capable of mediating survival responses

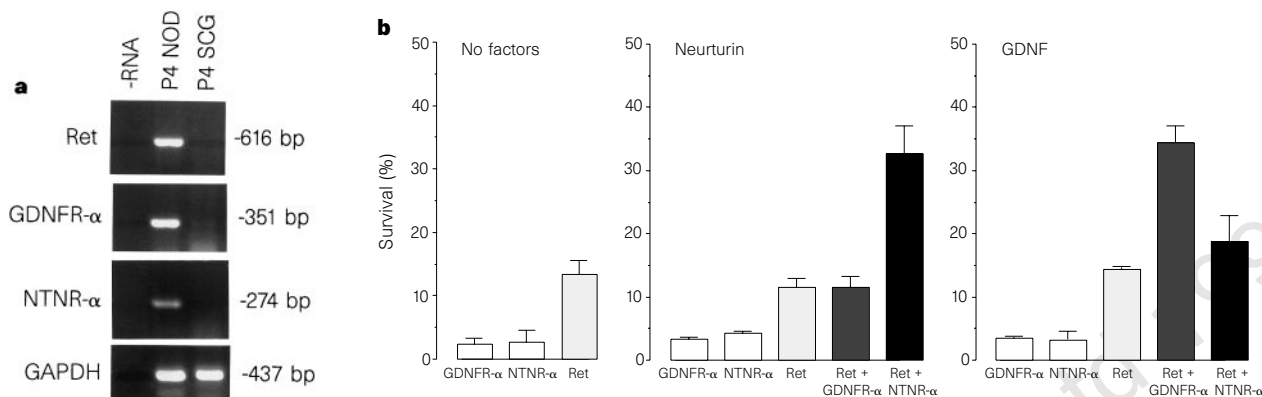


Figure 2 a, RT-PCR detection of endogenous *c-Ret*, *GDNFR- α* and *NTNR- α* transcripts in mouse P4 SCG neurons (which do not respond to GDNF and neurturin) and P4 mouse nodose neurons (which do respond to these factors). The presence of similar amount of the total RNA in samples is demonstrated by amplification of the 437-bp fragment of *GAPDH* mRNA. **b**, Survival of P4 mouse

SCG neurons 48 h after injection with expression plasmids for *GDNFR- α* and *NTNR- α* and *Ret*, singularly and in combination. The injected neurons were incubated in medium containing either neurturin or GDNF. Results are means ($\pm$ s.e.) of the combined results of 8 separate experiments ($n \geq 4$ for each experiment condition).

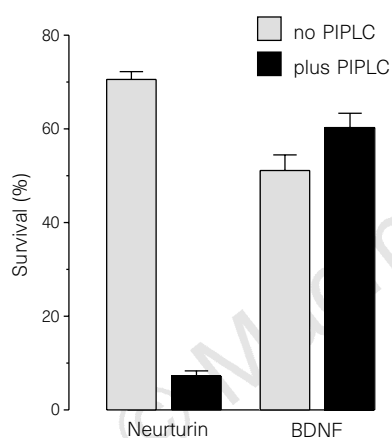


Figure 3 Survival of E6 chicken nodose ganglion neurons grown with either neurturin or BDNF for 36 h. Half of the cultures were treated with PIPLC. Results show mean ($\pm$ s.e.) percentage survival ($n = 4$).

to GDNF and neurturin. However, neurons coexpressing *NTNR- α* plus *Ret* had a substantially enhanced survival response to neurturin which was significantly greater than that of neurons expressing *Ret* alone ($P = 0.003$, t -test, $n = 6$). Similarly, neurons coexpressing *GDNFR- α* plus *Ret* had a substantially enhanced survival response to GDNF which was significantly greater than that of neurons expressing *Ret* alone ($P = 0.0002$, t -test, $n = 8$). In contrast, neurons coexpressing *GDNFR- α* plus *Ret* showed no enhanced survival response to neurturin; there were no more neurons expressing *Ret/GDNFR- α* surviving with neurturin than neurons expressing *Ret*. Similarly, the number of neurons expressing *Ret/NTNR- α* surviving with GDNF was not significantly different from the number of neurons expressing *Ret* surviving with this factor ($P > 0.2$, t -test, $n = 8$). These results imply that *GDNFR- α* and *NTNR- α* are receptors for GDNF and neurturin, respectively, and that *Ret* is required for signalling and the survival response of neurons to both factors. Although *GDNFR- α* and *NTNR- α* in the presence of *Ret* conferred specific survival responses to GDNF and neurturin at concentrations up to 5 ng ml^{-1} , at 10-fold higher concentrations of these factors some cross-talk in responsiveness was observed (see Supplementary Information).

To investigate further whether a GPI-linked protein is normally required for the survival response of neurons to neurturin, we treated neurturin-responsive embryonic chicken nodose ganglion neurons with phosphoinositide-specific phospholipase C (PIPLC), an enzyme that specifically cleaves GPI linkages¹⁶. PIPLC treatment

reduced the number of neurons surviving with neurturin from over 70% to less than 10% (Fig. 3). This was not due to a neurotoxic effect of PIPLC, because the survival response of nodose neurons to brain-derived neurotrophic factor (BDNF), which signals through the transmembrane *TrkB* receptor tyrosine kinase¹⁷, was unaffected by PIPLC treatment (Fig. 3). Similarly, the neurturin survival response of P4 SCG neurons coexpressing *Ret* and *NTNR- α* was significantly reduced from $30.6 \pm 2.9\%$ to $9.2 \pm 3.0\%$ by PIPLC treatment ($P < 0.01$, t -test, $n = 4$). The survival of these PIPLC-treated neurons was not significantly different from the survival of neurons expressing *Ret* alone ($13.3 \pm 2.2\%$, $P > 0.05$). This indicates that PIPLC treatment had eliminated the specific neurturin survival response in these neurons, which in turn demonstrates that ectopically expressed *NTNR- α* is required for this response.

Northern blotting was used to study the expression of *GDNFR- α* and *NTNR- α* transcripts in chicken embryos at different developmental stages (Fig. 4). A 10-kilobase (kb) *GDNFR- α* transcript and 3.4-kb and 2.8-kb *NTNR- α* transcripts were expressed in the nervous system and several other tissues. Regional and developmental differences in the levels of these transcripts were apparent in these tissues.

In summary, we have identified a specific GPI-linked receptor for neurturin that is structurally related to *GDNFR- α* . We have shown that the survival responses of neurons to neurturin and GDNF require *NTNR- α* and *GDNFR- α* , respectively, together with a common transmembrane receptor tyrosine kinase, *Ret*. Our

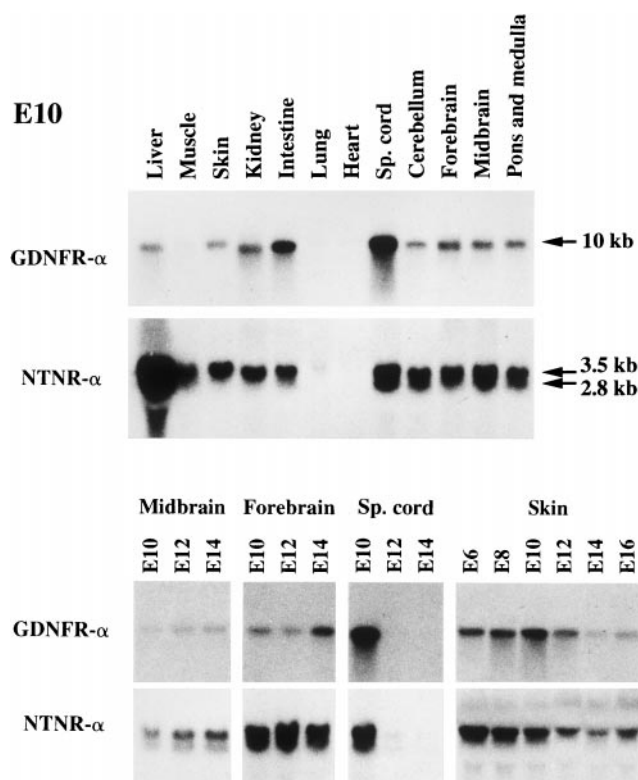


Figure 4 Analysis of the expression of *GDNFR-α* and *NTNR-α* mRNAs by northern blotting. Similar amounts of total RNA from various embryonic tissues at E10 (top) and skin and regions of the central nervous system (bottom) were transferred to filters and hybridized sequentially with *GDNFR-α* and *NTNR-α* probes. Bands corresponding to the 10-kb *GDNFR-α* and 3.5-kb and 2.8-kb *NTNR-α* transcripts are indicated. Sp. Cord, spinal cord.

demonstration that *NTNR-α* and *GDNFR-α* are required for ligand discrimination in the physiological responses of neurons to neurturin and GDNF is consistent with the results of binding studies which show that *NTNR-α* and *GDNFR-α* are specific high-affinity receptors for neurturin and GDNF, respectively¹⁸. Furthermore, because we investigated the actions of mammalian neurturin and GDNF on avian receptors, our study also shows that the specific, functional interactions between these neurotrophic factors and their corresponding GPI-linked receptors have been conserved between two classes of vertebrates. Previous studies have identified several unrelated GPI-linked, ligand-binding components of multicomponent receptors, such as the CNTF receptor¹⁵ and the bacterial endotoxin receptor^{19,20}. However, we have established the existence of a family of GPI-linked proteins that discriminate between structurally related ligands in multicomponent receptors that share a common transmembrane signalling component. □

Methods

Receptor cloning and expression. An E10 chicken brain cDNA library in λZAPII was screened²¹ using a mouse GDNF cDNA probe at low stringency (hybridization at 59 °C, final wash at 65 °C in 2X SSC, 0.2% SDS). Five *GDNFR-α* and three *NTNR-α* cDNA independent clones were sequenced. Northern blot analysis²¹ used unique regions of the *GDNFR-α* and *NTNR-α* cDNAs as specific probes. For RT-PCR analysis of *GDNFR-α*, *NTNR-α* and *c-Ret* mRNA expression in cultured mouse neurons, total RNA was reverse transcribed and amplified²². The amounts of total RNA in samples were normalized by amplification of a 437-bp fragment of glyceraldehyde-3-phosphate dehydrogenase mRNA²². The primers were as follows: *c-Ret*, 5'-TGTATGTAGAC-CAGCCAGCT-3' and 5'-ACTATGCACAAAGCCTCCAG-3'; *GDNFR-α*, 5-

CATGTTCTAGCCACTCTGT-3' and 5'-TCCAGTAGGTCATTTCCCTG-3'; *NTNR-α*, 5'-ATGATCTTGGCAAACGCCCTTCTG-3' and 5'-GCTTCATA-GAACTCCTCACCCTC-3'. Amplification was carried out for 40 cycles of 95 °C for 45 s; 54 °C for 30 s; and 72 °C for 60 s.

Neuronal culture and microinjection studies. Purified P4 SCG neurons were grown in defined medium²³. After incubation for 12 h with NGF (2 ng ml⁻¹), the neurons were washed extensively to remove NGF and injected with expression plasmids into the nucleus (*GDNFR-α* cDNA and *NTNR-α* cDNA in pMEX, and *c-Ret* cDNA in pRC/CMV) as described²⁴. The cultures were supplemented with either GDNF or neurturin immediately after injection, and the number of surviving neurons was counted 48 h later and is expressed as a percentage of the number injected. To investigate the importance of GPI-linked proteins in neurturin responsiveness, neurturin-responsive E6 chicken nodose neurons were cultured in 11-mm diameter wells²⁵. BDNF (5 ng ml⁻¹) or neurturin (5 ng ml⁻¹) was added to the cultures 1.5 h after plating, and half of the cultures were supplemented with PIPLC (4 μg ml⁻¹) at the time of plating and after a further 12 and 24 h (ref. 9). The number of surviving neurons was counted at 36 h, and is expressed as a percentage of the number plated.

Received 7 March; accepted 19 May 1997.

- Kotzbauer, P. T. et al. Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* **384**, 467–470 (1996).
- Lin, L. H., Doherty, D. H., Lile, J. D., Bektesh, S. & Collins, F. GDNF: A glial cell-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130–1132 (1993).
- Henderson, C. E. et al. GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266**, 1062–1064 (1994).
- Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A. & Davies, A. M. GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15**, 821–828 (1995).
- Trupp, M. et al. Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J. Cell Biol.* **130**, 137–148 (1995).
- Moore, M. W. et al. Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76–79 (1996).
- Pichel, J. G. et al. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73–76 (1996).
- Sanchez, M. P. et al. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70–73 (1996).
- Treanor, J. et al. Characterization of a receptor for glial cell line-derived neurotrophic factor. *Nature* **382**, 80–83 (1996).
- Jing, S. et al. GDNF-induced activation of the ret protein tyrosine kinase is mediated by *GDNFR-α*, a novel receptor for GDNF. *Cell* **85**, 1113–1124 (1996).
- Trupp, M. et al. Functional receptor for GDNF encoded by the *c-ret* proto-oncogene. *Nature* **381**, 785–789 (1996).
- Durbec, P. et al. GDNF signalling through the Ret receptor tyrosine kinase. *Nature* **381**, 789–793 (1996).
- Vega, C. C., Worby, C. A., Lechner, M. S., Dixon, J. E. & Dressler, G. R. Glial cell line-derived neurotrophic factor activates the receptor tyrosine kinase RET and promotes kidney morphogenesis. *Proc. Natl Acad. Sci. USA* **93**, 10657–10661 (1996).
- Gerber, L. D., Kodukula, K. & Udenfried, S. Phosphatidylinositol glycan (PI-G) anchored membrane proteins. Amino acid requirements adjacent to the site of cleavage and PI-G attachment in the COOH-terminal signal peptide. *J. Biol. Chem.* **267**, 12168–12173 (1992).
- Davis, S. et al. LIFRβ and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science* **260**, 1805–1808 (1993).
- Koke, J. A., Yang, M., Henner, D. J., Volwerk, J. J. & Griffin, O. H. High-level expression in *Escherichia coli* and rapid purification of phosphatidylinositol-specific phospholipase C from *Bacillus cereus* and *Bacillus thuringiensis*. *Protein Expr. Purif.* **2**, 51–58 (1991).
- Klein, R. et al. The TrkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66**, 395–403 (1991).
- Klein, R. D. et al. A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. *Nature* **387**, 717–721 (1997).
- Lee, J. D. et al. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. *Proc. Natl Acad. Sci. USA* **90**, 9930–9934 (1993).
- Pugin, J. et al. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc. Natl Acad. Sci. USA* **90**, 2744–2747 (1993).
- Baka, I. D. et al. Intracellular compartmentalisation of two differently spliced S-REX/NSP mRNAs in neurons. *Mol. Cell Neurosci.* **7**, 289–303 (1996).
- Ninkina, N. et al. Expression and function of TrkB variants in developing sensory neurons. *EMBO J.* **15**, 6385–6393 (1996).
- Davies, A. M., Minichiello, L. & Klein, R. Developmental changes in NT3 signalling via TrkA and TrkB in embryonic neurons. *EMBO J.* **14**, 4482–4489 (1995).
- Allsopp, T. E., Wyatt, S., Paterson, H. F. & Davies, A. M. The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. *Cell* **73**, 295–306 (1993).
- Buj-Bello, A., Pinon, L. G. & Davies, A. M. The survival of NGF-dependent but not BDNF-dependent cranial sensory neurons is promoted by several different neurotrophins early in their development. *Development* **120**, 1573–1580 (1994).

Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

Acknowledgements. We thank H. Philips, R. Klein, R. Vanden, B. Moffet and L. Simmons for the purified recombinant GDNF and neurturin; J. Winslow and G. Burdon for the purified recombinant BDNF; and D. Roche and J. Allan for preparing the illustrations. This work was supported by Action Research and the Wellcome Trust.

Correspondence and requests for materials should be addressed to A.M.D. (e-mail: amd2@st-and.ac.uk).

Gln 63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor-1

Gudula Schmidt*, Peter Sehr*, Matthias Wilm†, Jörg Selzer*, Matthias Mann† & Klaus Aktories*

* Institut für Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, Hermann-Herder-Strasse 5, 79104 Freiburg, Germany

† EMBL, 69012 Heidelberg, Germany

The actin cytoskeleton is regulated by GTP-hydrolysing proteins, the Rho GTPases^{1,2}, which act as molecular switches in diverse signal-transduction processes³. Various bacterial toxins can inactivate Rho GTPases by ADP-ribosylation¹ or glucosylation⁴. Previous research has identified Rho proteins as putative targets for *Escherichia coli* cytotoxic necrotizing factors 1 and 2 (CNF1 and 2)^{5,6}. These toxins induce actin assembly and multinucleation in culture cells. Here we show that treatment of RhoA with CNF1 inhibits the intrinsic GTPase activity of RhoA and completely blocks GTPase activity stimulated by the Rho-GTPase-activating protein (rhoGAP). Analysis by mass spectrometry and amino-acid sequencing of proteolytic peptides derived from CNF1-treated RhoA indicate that CNF1 induces deamidation of a glutamine residue at position 63 (Gln 63) to give constitutively active Rho protein.

The cytotoxic necrotizing factors CNF1 and CNF2 (each of M_r 115K) are produced by up to half of the different *E. coli* strains isolated from extra-intestinal infections, and by up to a fifth of *E. coli* strains from diarrhoea^{7,8}. These toxins cause tissue damage and death of the animal host⁸. CNFs induce actin polymerization and increase the F-actin content of cells⁶. They inhibit cytokinesis, cause formation of multinucleated cells, and induce membrane ruffling⁹. Because treatment of intact cells with CNFs changes the migration of Rho on SDS-PAGE, it has been suggested that CNFs act on Rho^{5,6}.

We expressed and purified CNF1 as a fusion protein with glutathione S-transferase (GST) and tested the activity of the recombinant toxin in NIH3T3 cells. GST–CNF1 (at 300 ng ml^{−1}) induced multinucleation of ~90% of cells after 24 h of treatment. Moreover, staining of the actin cytoskeleton with rhodamine-phalloidin revealed a dense network of actin fibres resembling that induced by CNF1 (Fig. 1a–d).

Although major morphological changes in cells (multinucleation) were not observed earlier than 12–16 h after intoxication, treatment of cells with GST–CNF1 at 300 ng ml^{−1} for only 2–3 h altered the migration of Rho on SDS-PAGE. As shown in Fig. 2a, GST–CNF1 caused a shift in the apparent molecular mass of Rho protein labelled by C3-catalysed [³²P]ADP-ribosylation, indicating that the GTPase has been covalently modified. This change in migration induced by GST–CNF1 was also observed after ¹⁴C-glucosylation of Rho by *Clostridium difficile* toxin B (not shown).

Treatment of recombinant RhoA with GST–CNF1 (molar ratio 16:1) for 3 h at 37°C caused a similar shift in the apparent molecular mass of [³²P]ADP-ribosylated RhoA on SDS-PAGE, as

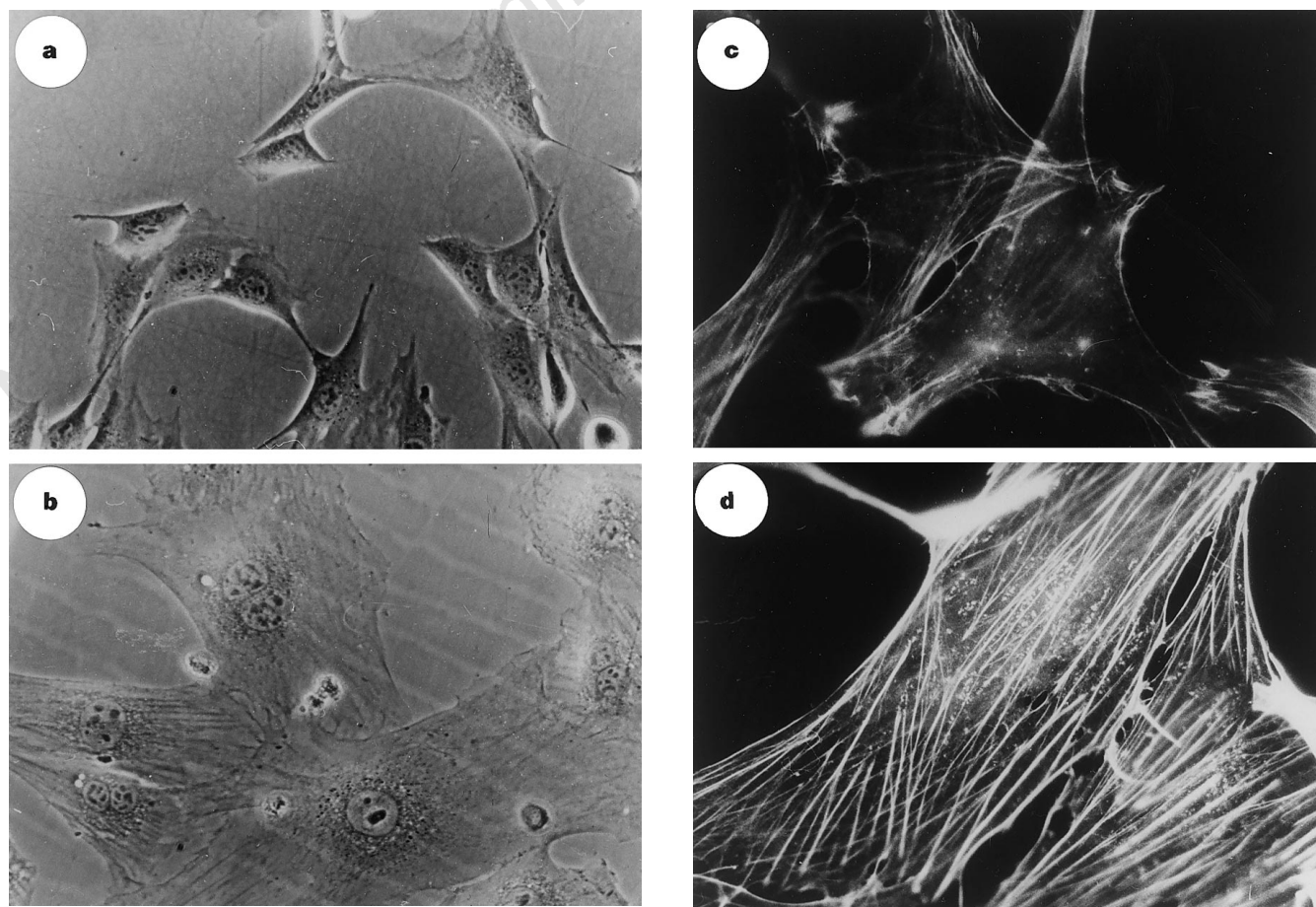


Figure 1 Induction of multinucleation and increase in F-actin by CNF1. NIH3T3 cells were treated without (a, c) and with CNF1 (300 ng ml^{−1}; b, d) for 16 h. Cells were then analysed by phase-contrast microscopy (a, b) or the actin cytoskeleton

was stained with rhodamine-labelled phalloidin for fluorescence microscopy (c, d).

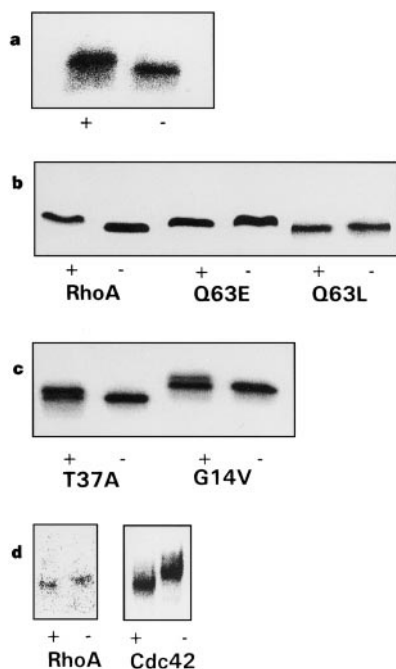


Figure 2 Effects of CNF1 on the migration behaviour of Rho. **a**, Cells were treated without (–) and with (+) GST–CNF1 (300 ng ml^{–1}) for 16 h. **b**, **c**, WT-RhoA (RhoA), mutant Gln63Glu-RhoA (Q63E), Gln63Leu-RhoA (Q63L), Thr37Ala-RhoA (T37A) and Gly14Val-RhoA (G14V) were treated without (–) and with GST–CNF1 (+) at a ratio of 10:1 for 3 h. Cell lysates and recombinant Rho proteins were [³²P]ADP-ribosylated by transferase C3. Labelled proteins were analysed by SDS–PAGE and phosphorimaging. **d**, RhoA and Cdc42 were treated without (–) and with GST–CNF1 (+) at a ratio of 16:1 and 2:1, respectively, for 3 h. Thereafter, GTPases were [¹⁴C]-glucosylated by *C. difficile* toxin B and analysed by non-denaturing gel electrophoresis and phosphorimaging.

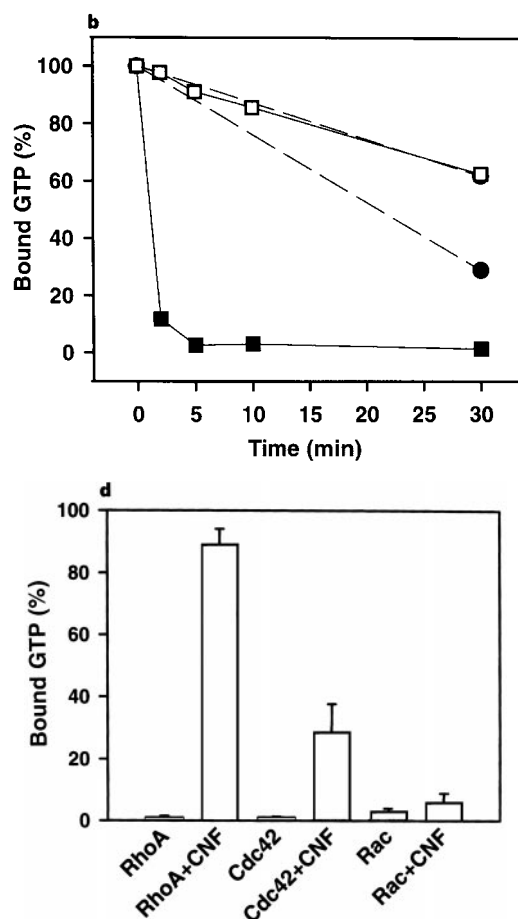
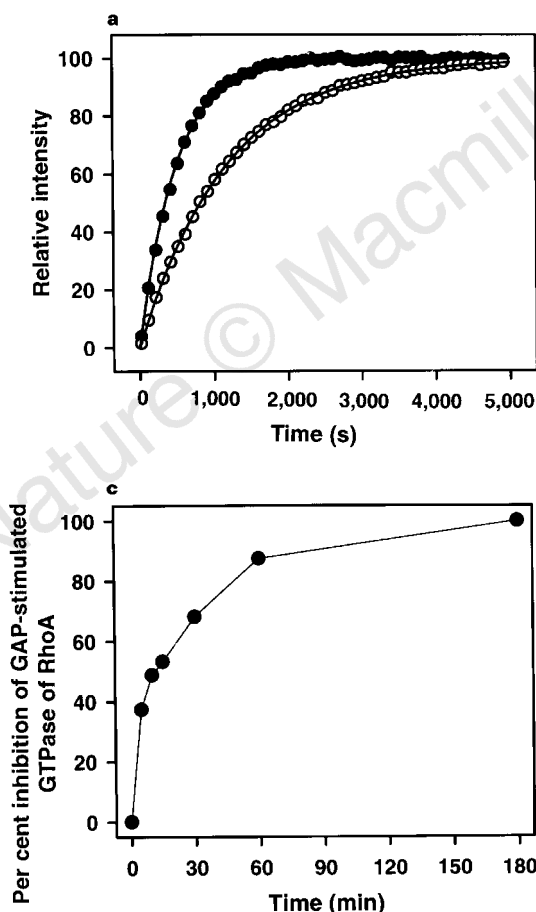


Figure 3 a, Nucleotide-binding of CNF1-treated RhoA. Binding of mantGDP to RhoA previously treated without (●) and with (○) GST–CNF1 (ratio of RhoA: CNF1 was 16:1) was followed as the increase in fluorescence intensity. **b**, GTPase activity of CNF1-treated RhoA. RhoA was treated without (●, ■) and with GST–CNF1 (□, ○) at a ratio of 16:1. Thereafter, [³²P]GTP was bound and the GTPase incubated in the absence (●, ○) and presence of rhoGAP (40 nM; ■, □) for the times given (final concentrations of Rho proteins, 0.8 μM). Radioactivity remaining on the GTPase was determined in a filter-binding assay as described in Methods.

c, Time-dependent inhibition of GAP-stimulated GTP hydrolysis by CNF1. RhoA was treated with GST–CNF1 (ratio 16:1) for the indicated times. The inhibition of GAP-stimulated GTPase activity of RhoA is shown as per cent of maximum. Without CNF treatment, the remaining radioactivity was 97% and 16% in the absence and presence of GAP, respectively. **d**, Influence of CNF1 on stimulation of GTPase activity of RhoA, Rac1 and Cdc42 by rhoGAP. RhoA, Rac1 and Cdc42 were treated with GST–CNF1 at a ratio of 16:1.

after treatment of intact cells with the toxin (Fig. 2b). In contrast, when RhoA was incubated with heat-inactivated GST–CNF1 (15 min at 95 °C) or without toxin, the migration of the GTPase was unaffected (results not shown).

Next we studied the effects of GST–CNF1 on nucleotide-binding of RhoA by monitoring the increase in fluorescence intensity of mantGDP (see Methods)¹⁰. As shown in Fig. 3a, total mantGDP binding was the same as for the control and CNF-treated RhoA, indicating that the GTPase structure was native. MantGDP binding (indicative of the release of previously bound nucleotide) was about twofold slower after toxin treatment. Figure 3b shows the influence of CNF1 on rhoGTPase activity. Basal GTPase activity of control RhoA was stimulated ~20-fold by p50 rhoGAP protein (M_r 50K) at 40 nM. Treatment of RhoA with GST–CNF1 decreased the basal GTPase activity and completely blocked GAP-stimulated GTP hydrolysis. We checked that this effect was not due to changes in buffer or cation concentrations. Heat inactivation completely blocked CNF1 effects on Rho GTPase activity (not shown). The time course (Fig. 3c) shows that inhibition of GAP-stimulated GTPase activity was half-maximal after GST–CNF1 treatment of RhoA for 10 min; inhibition was complete after 2–3 h.

Next we studied the effects of CNF1 on GAP-stimulated GTP hydrolysis by the Rho GTPases Cdc42 and Rac1. Whereas CNF1 (at a molar ratio of GTPase to CNF1 of 16:1) part inhibited GAP stimulation of Cdc42 (~30%), Rac1 GTPase activity was hardly affected by CNF1 treatment at this toxin concentration, suggesting

that CNF1 acts preferentially with Rho (Fig. 3d). At higher concentrations of CNF1 (molar ratio of GTPase to CNF1 of 2:1), the GAP-stimulated GTPase activity of Rho, Cdc42 and Rac was inhibited by >90, 60 and 38%, respectively. We checked a possible covalent modification of the GTPase on SDS–PAGE. No change in the migration of CNF1-treated Cdc42 was detected on SDS–PAGE, but treated Cdc42 migrated faster than controls on non-denaturing gels (Fig. 2d). CNF-modified RhoA behaved like Cdc42 (Fig. 2d) (Rac gave no clear band in non-denaturing gels).

To identify the structural changes induced by CNF1 that were responsible for inhibiting GTPase activity and activating Rho, we used mass spectrometric (MS) analysis. Measurements of control and CNF1-treated RhoA showed an upward shift in mass for the toxin-treated GTPase of 1 to 3 daltons. Comparison of the tryptic peptide maps of the two proteins revealed a 2K peptide whose mass was shifted by 1 dalton between the two proteins (from 2.008K to 2.009K for CNF1-treated RhoA). The doubly charged ion was sequenced by tandem mass spectrometry. The fragment spectrum of the peptide from the untreated RhoA corresponded to the sequence from Gln 52 through to Arg 68 (QVELALWDTAGQEDYDR). The tandem MS spectrum of CNF1-treated RhoA showed that the Gln 63 residue was deamidated to glutamic acid (Fig. 4), thereby explaining the observed mass difference between the two peptides. Moreover, a toxin-induced deamidation is consistent with the finding that *in vitro* modification of Rho by CNF did not depend on any added cofactor such as NAD⁺

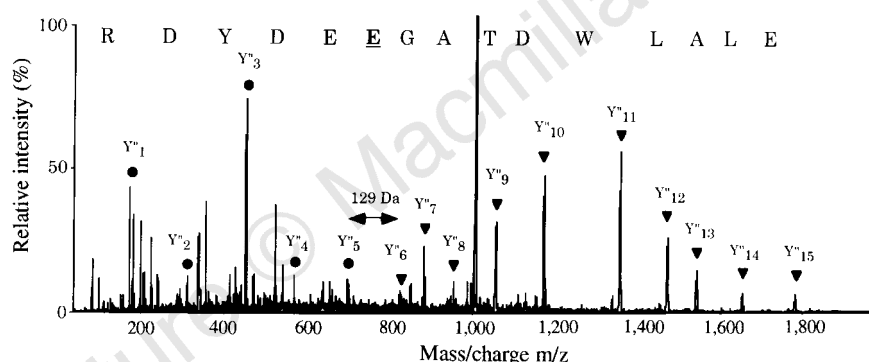


Figure 4 Tandem MS spectrum of the doubly charged tryptic peptide Gln52–Arg68 of the CNF I-exposed RhoA. The sequence of the peptide is reflected by the continuous Yⁿ ion series, the C-terminal sequence ions of the peptide. The mass difference between the Y₆ⁿ and Y₅ⁿ ion is 129 daltons, corresponding to a glutamic acid. When comparing the tandem MS spectra of the peptides from untreated and CNF I-treated RhoA the Y₆ⁿ–Y₅ⁿ ions are at the same position (marked by ● in the spectrum), whereas the Y₆ⁿ–Y₅ⁿ ions are shifted upwards by 1 dalton (▼), indicative of the deamidation of Gln63.

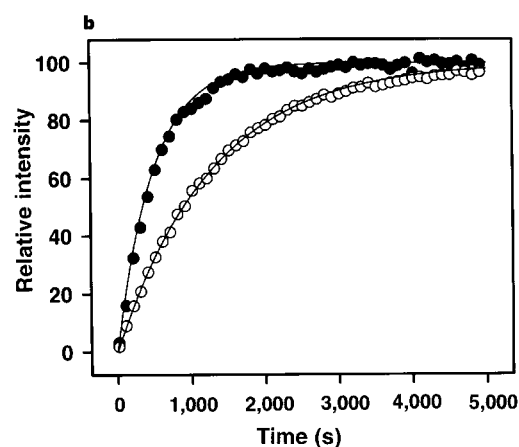
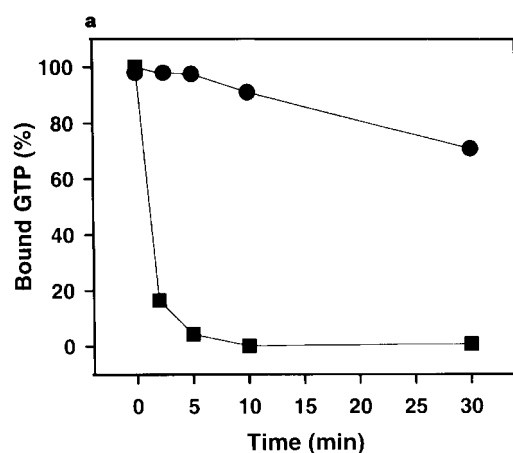


Figure 5 GTPase activity and nucleotide-binding of Gln63Glu-RhoA. **a**, [γ -³²P]GTP was bound to Gln63Glu-RhoA (●) or wild-type RhoA (■); each at a final concentration of 0.8 μ M) and the GTPases were incubated with p50 rhoGAP (40 nM) for the times indicated. Radioactivity remaining on the GTPase was determined in a

filter-binding assay as described in Methods. **b**, Time course of binding of mantGDP (showing the release of prebound nucleotide) to wild-type RhoA (●) or Gln63Glu-RhoA (○) was followed by the increase in mant-fluorescence intensity.

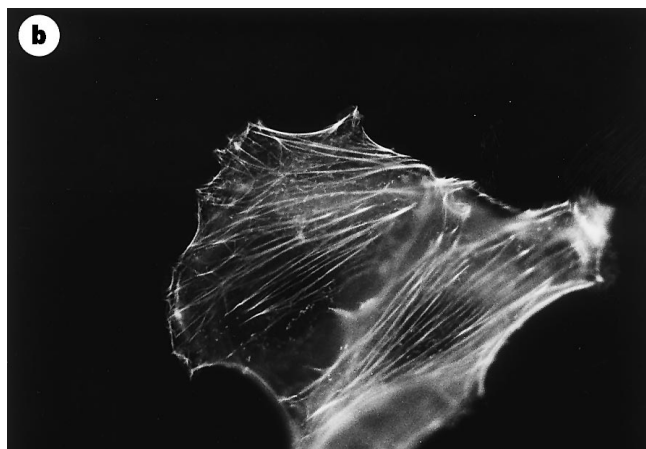
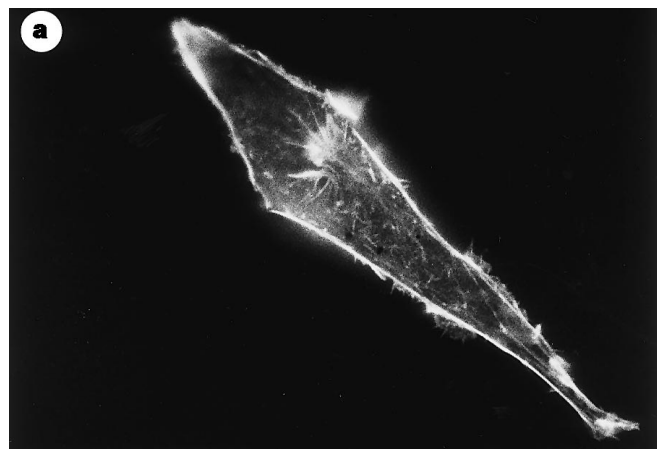


Figure 6 Induction of stress fibres by microinjection of Gln63Glu-RhoA. To avoid wild-type RhoA-induced effects on the actin cytoskeleton, CAAX-box-deleted (Δ CLVL) proteins were used for microinjection. Recombinant Gln63Glu-RhoA protein (2 mg ml^{-1} ; **b**) but not the control RhoA (2 mg ml^{-1} ; **a**) induced formation of

or UDP-glucose, which are essential for modification of the GTPase by C3-like ADP-ribosyltransferases and clostridial glucosyltransferases, respectively.

To corroborate a role of Gln 63 in CNF1-induced covalent modification of RhoA, we constructed a Gln63Glu mutant of RhoA, in which a glutamate residue is substituted for glutamine at position 63, expressed it in *E. coli*, then purified and tested it as a substrate for CNF1. As shown in Fig. 2b, treatment of Gln63Glu-RhoA with GST-CNF1 did not cause a shift in the SDS-PAGE pattern. Even without toxin treatment, this protein migrated slightly more slowly than wild-type RhoA. Similarly, there was no change in migration after treatment of Gln63Leu-RhoA with CNF1. In contrast, a shift was induced by CNF1 in the control mutant proteins Thr37Ala- and Gly14Val-RhoA (Fig. 2c). As for CNF1-treated RhoA, GAP stimulation of GTP hydrolysis was blocked in the mutant Gln63Glu-RhoA (Fig. 5a) and mantGDP binding of this mutant was slowed by a factor of two (preloading of the GTPase with GDP did not change this effect) (Fig. 5b). Moreover, microinjection of Gln63Glu-RhoA into NIH3T3 cells induced the formation of stress fibres (Fig. 6), as reported for the Gln63Leu-RhoA mutation¹¹. We did not observe multinucleation after microinjection of Gln63Glu-RhoA even after prolonged incubation (24 h). This discrepancy in the effect of CNF in intact cells could be explained by modification of other Rho subfamily proteins (for example, Cdc42).

Gln 63 of Rho is essential for GTPase activity. For example, mutation of this residue to leucine inhibits GTPase, blocks GAP stimulation and induces dominant positive activity of the GTPase^{12,13}. In Ras, the function of the equivalent residue (Gln 61) has been studied in more detail. Mutation of Gln 61 to almost any other amino acid blocks both intrinsic and GAP-stimulated GTPase activity^{14,15}. This glutamine residue in Ras may act either as a general base to activate water for nucleophilic attack or it could be involved in stabilizing the transition state of the GTPase reaction¹⁵; Gln 63 in Rho may have a similar role. Thus deamidation of this pivotal glutamine residue, resulting in the formation of constitutively active Rho proteins, is a new mode of action for intracellularly acting toxins.

Methods

Materials. [³²P]NAD⁺ and UDP-[¹⁴C]glucose were obtained from DuPont NEN. Wild-type RhoA, Rac1, Cdc42, Gly14Val-RhoA, Thr37Ala-RhoA, Gln63Leu-RhoA and p50 rhoGAP (the latter two expression constructs were from A. Hall) were prepared from their fusion proteins (for example, RhoA-

actin stress fibres after microinjection into NIH3T3 cells. One hour after microinjection, cells were fixed and stained for F-actin by rhodamine-conjugated phalloidin. The fluorescence micrograph is shown.

GST) as described¹⁶. Gln63Glu-RhoA and truncated Δ CLVL Rho proteins were generated using a polymerase chain reaction (PCR)-based mutagenesis system and the proper DNA sequence was checked. *N*-methylanthraniloyl GDP (mantGDP) was prepared as described¹⁷.

Cloning and purification of GST-CNF1. The CNF1 gene with flanking *Bam*HI and *Eco*R1 sites was generated by PCR from the vector PISS 391 (ref. 18; a gift from J. Hacker) and cloned in-frame into the expression vector pGEX2TGL + 2. The proper construct was checked by DNA sequencing. The GST fusion protein was isolated by affinity chromatography with glutathione-Sepharose (Pharmacia).

Cell culture. NIH3T3 cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum in humidified CO₂ (5%) at 37 °C. After washing with PBS, cells were scraped off and lysed by sonication in lysis buffer (20 mM TEA, pH 7.4, 2 mM MgCl₂, 2 mM EDTA, 1 mM PMSE, 0.4 mg ml⁻¹ aprotinin and 0.4 mg ml⁻¹ benzamide). Cell debris and whole cells were eliminated by centrifugation (30 min, 14,000 r.p.m.) and the protein concentration of the cell lysate was determined. For actin staining, cells were treated for 16–24 h with GST-CNF1 (300 ng ml⁻¹).

Actin staining. CNF1-treated and control cells were washed 3 times with ice-cold PBS, pH 7.4, and fixed with 4% formaldehyde, 0.1% Tween 20 in PBS at room temperature for 10 min. After intensive washing with PBS, cells were incubated with rhodamine-conjugated phalloidin (1 unit per coverslip) at room temperature in a humidified atmosphere for 60 min, washed again, and applied for fluorescence microscopy.

Microinjection. For microinjection, CAAX-box-deleted recombinant wild-type RhoA and Gln63Glu-RhoA (2 mg ml^{-1} each) were microinjected into NIH3T3 cells with a Microinjector 5242 (Eppendorf). After 1 h, cells were fixed and stained for F-actin by rhodamine-phalloidin as described.

ADP-ribosylation and glucosylation assays. For ADP-ribosylation¹⁹, cell lysates were adjusted to the same protein concentration (1.5 mg ml^{-1}). Lysates or purified proteins ($30 \text{ } \mu\text{g ml}^{-1}$) were incubated with *C. botulinum* exoenzyme C3 ($0.5 \text{ } \mu\text{g ml}^{-1}$) and $1 \text{ } \mu\text{M}$ [³²P]NAD⁺ ($\sim 0.1 \text{ } \mu\text{Ci per tube}$) in $50 \text{ } \mu\text{l}$ ADP-ribosylation buffer (25 mM triethanolamine-HCl, pH 7.5, 2 mM MgCl₂, 1 mM DTT) at 37 °C for 15 min. The reaction was terminated by addition of $10 \text{ } \mu\text{l}$ stop reagent (10% SDS, 10 mM DTT) and heated for 5 min at 95 °C. Thereafter, $20 \text{ } \mu\text{l}$ 15 mM *N*-ethylmaleimide was added and the samples incubated at room temperature for 15 min. Proteins were analysed by SDS-PAGE and phosphorimaging (Molecular Dynamics). For glucosylation recombinant proteins ($50 \text{ } \mu\text{g ml}^{-1}$) or cell lysates (1.5 mg ml^{-1} protein) were incubated with toxin B ($1 \text{ } \mu\text{g ml}^{-1}$) in a buffer containing $10 \text{ } \mu\text{M}$ UDP-[¹⁴C]glucose, 10 mM HEPES (pH 7.4) and 150 mM KCl for 30 min at 37 °C. Glucosylated proteins were analysed by SDS-PAGE or by non-denaturing gel electrophoresis and phosphorimaging as described.²⁰

In vitro modification of GTPases by CNF1. Small GTPases were treated

essentially as described for modification of Rho with dermonecrotic toxin (DNT)²¹. In general, recombinant proteins were incubated with GST–CNF1 in a molar ratio of 10:1 or 16:1 (GTPase:GST–CNF1) (or as indicated) in a reaction buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM EDTA for 3 h at 37 °C.

Nucleotide-binding assay. RhoA was modified as described above but with GST–CNF1 immobilized to glutathione–Sepharose beads to remove the toxin after the reaction. 0.5 µM RhoA, CNF1-modified RhoA or Gln63Glu–RhoA were incubated in buffer C (150 mM NaCl, 2.5 mM MgCl₂, 10 mM triethanolamine–HCl, pH 7.5) at 37 °C. After addition of 2 µM mantGDP, the increase in mant-fluorescence upon binding to RhoA was monitored in a Perkin Elmer LS-50B luminescence spectrometer. The emission was measured at 444 nm with excitation at 357 nm.

GTPase assay. Recombinant Rho proteins modified by CNF1 were loaded with [γ -³²P]GTP for 5 min at 37 °C in loading buffer (10 mM EDTA, 2 mM DTT, 50 mM Tris–HCl, pH 7.5). MgCl₂ was then added to 12 mM. Unbound nucleotide was removed by gel filtration or unlabelled GTP (5 mM) was added. For GAP stimulation, 40 nM of p50 rhoGAP was added (Rho at 0.8 µM). GTPase activities were determined by measuring the loss of protein-bound radioactivity in a filter-binding assay¹.

Mass spectrometric analysis. All electrospray experiments were done on a triple quadrupole instrument (API III, Perkin–Elmer). CNF1-exposed and untreated RhoA were desalted on a 100 nl RI Poros microcolumn as described²². They were eluted in 2 × 0.5 µl 50% acetonitrile, 5% formic acid into a nano electrospray spraying needle for mass spectrometry.

The proteins were digested with trypsin (Boehringer Mannheim, sequencing grade) for 5 h. The peptide mixture was step-eluted with 2 × 0.4 µl 50% methanol, 5% formic acid into the emitter of the nano electrospray ion source.

Received 14 March; accepted 17 April 1997.

1. Paterson, H. F. Microinjection of recombinant p21^{tho} induces rapid changes in cell morphology. *J. Cell Biol.* **111**, 1001–1007 (1990).
2. Machesky, L. M. & Hall, A. Rho: A connection between membrane receptor signalling and the cytoskeleton. *Trends Cell Biol.* **6**, 304–310 (1996).
3. Lim, L., Manser, E., Leung, T. & Hall, C. Regulation of phosphorylation pathways by p21 GTPases—the p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur. J. Biochem.* **242**, 171–185 (1996).
4. Just, I. *et al.* Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* **375**, 500–503 (1995).
5. Oswald, E. *et al.* Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proc. Natl Acad. Sci. USA* **91**, 3814–3818 (1994).
6. Fiorentini, C. *et al.* *Escherichia coli* cytotoxic necrotizing factor 1: Evidence for induction of actin assembly by constitutive activation of the p21 Rho GTPase. *Infect. Immun.* **63**, 3936–3944 (1995).
7. Caprioli, A., Falbo, V., Roda, L. G., Ruggeri, F. M. & Zona, C. Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological cell alterations. *Infect. Immun.* **59**, 1300–1306 (1991).
8. de Rycke, J. *et al.* Evidence for two types of cytotoxic necrotizing factor in human and animal clinical isolates of *Escherichia coli*. *J. Clin. Microbiol.* **28**, 694–699 (1990).
9. Falzano, L. *et al.* Induction of phagocytic behaviour in human epithelial cells by *Escherichia coli* cytotoxic necrotizing factor type 1. *Mol. Microbiol.* **9**, 1247–1254 (1993).
10. Leonard, D. A., Evans, T., Hart, M., Cerione, R. A. & Manor, D. Investigation of the GTP-binding/GTPase cycle of Cdc42Hs using fluorescence spectroscopy. *Biochemistry* **33**, 12323–12328 (1994).
11. Ridley, A. J. Microinjection of Rho and Rac into quiescent Swiss 3T3 cells. *Methods Enzymol.* **256**, 313–320 (1995).
12. Diekmann, D. & Hall, A. *In vitro* binding assay for interactions of Rho and Rac with GTPase-activating proteins and effectors. *Methods Enzymol.* **256**, 207–215 (1995).
13. Renshaw, M. W., Toksoz, D. & Schwartz, M. A. Involvement of the small GTPase Rho in integrin-mediated activation of mitogen-activated protein kinase. *J. Biol. Chem.* **271**, 21691–21694 (1996).
14. Der, C. J., Finkel, T. & Cooper, G. M. Biological and biochemical properties of human ras^H genes mutated at codon 61. *Cell* **44**, 167–176 (1986).
15. Schweins, T. *et al.* Substrate-assisted catalysis as a mechanism for GTP hydrolysis of p21^{ras} and other GTP-binding proteins. *Nature Struct. Biol.* **2**, 36–44 (1995).
16. Just, I. *et al.* *Clostridium difficile* toxin B acts on the GTP-binding protein Rho. *J. Biol. Chem.* **269**, 10706–10712 (1994).
17. Hiratsuka, T. New ribose-modified fluorescent analogs of adenine and guanine nucleotides available as substrates for various enzymes. *Biochim. Biophys. Acta* **742**, 496–508 (1983).
18. Falbo, V., Pace, T., Picci, L., Pizzi, E. & Caprioli, A. Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli*. *Infect. Immun.* **61**, 4909–4914 (1993).
19. Aktories, K. & Just, I. *In vitro* ADP-ribosylation of Rho by bacterial ADP-ribosyltransferases. *Methods Enzymol.* **256**, 184–195 (1995).
20. Just, I., Selzer, J., Von Eichel-Streiber, C. & Aktories, K. The low molecular mass GTP-binding protein Rho is affected by toxin A from *Clostridium difficile*. *J. Clin. Invest.* **95**, 1026–1031 (1995).
21. Horiguchi, Y., Senda, T., Sugimoto, N., Katahira, J. & Matsuda, M. *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein rho. *J. Cell Sci.* **108**, 3243–3251 (1995).
22. Wilm, M. & Mann, M. Analytical properties of the nano-electrospray ion source. *Anal. Chem.* **68**, 1–8 (1996).

Acknowledgements. We thank I. Just for critical reading of the manuscript.

Correspondence and requests for materials should be addressed to K.A. (e-mail: aktories@ruf.uni-freiburg.de).

Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine

Gilles Flatau*, Emmanuel Lemichez*, Michel Gauthier*, Pierre Chardin†, Sonia Paris†, Carla Fiorentini‡ & Patrice Boquet*

*INSERM U452, Faculté de Médecine, avenue de Valombrose, Nice 06107, Cedex 2, France

†Institut de Pharmacologie Moléculaire et Cellulaire du CNRS, route des Lucioles, Sophia Antipolis, Valbonne 06560, France

‡Department of Ultrastructures, Istituto Superiore di Sanità, viale Regina Elena, 00161, Roma, Italy

Pathogenic *Escherichia coli* are responsible for a variety of diseases, including diarrhoea, haemolytic uraemic syndrome, kidney infection, septicaemia, pneumonia and meningitis. Toxins called cytotoxic necrotizing factors (CNFs) are among the virulence factors produced by uropathogenic (CNF1)¹ or enteropathogenic (CNF2)² *E. coli* strains that cause diseases in humans and animals, respectively. CNFs induce an increase in the content of actin stress fibres and focal contacts in cultured cells^{3,4}. Effects of CNFs on the actin cytoskeleton correlated with a decrease in the electrophoretic mobility of the GTP-binding protein Rho^{4,5} and indirect evidence indicates that CNF1 might constitutively activate Rho⁶. Here we show that CNF1 catalyses the deamidation of a glutamine residue at position 63 of Rho, turning it into glutamic acid, which inhibits both intrinsic GTP hydrolysis and that stimulated by its GTPase-activating protein (GAP). Thus, this deamidation of glutamine 63 by CNF1 leads to the constitutive activation of Rho, and induces the reorganization of actin stress fibres. To our knowledge, CNF1 is the first example of a bacterial toxin acting by deamidation of a specific target protein.

Incubation of Vero cells with CNF1 induces actin stress fibre accumulation and cell spreading (Fig. 1a, b), with a concomitant decrease in the electrophoretic mobility of the Rho protein on SDS–PAGE (Fig. 1c). Incubation of recombinant RhoA with purified CNF1 *in vitro* gives the same mobility shift as that observed after *in vivo* treatment (Fig. 2). This result indicates that CNF1 directly modifies Rho without the need for cellular cofactors. Incubation with CNF1 did not modify the electrophoretic mobilities of Rac, Cdc42, Rab6 or Ras either *in vivo* or *in vitro* (data not shown). *In vitro* induction of the mobility shift by CNF1 was blocked by heat denaturation of either RhoA or CNF1 (Fig. 2). To analyse the modification induced by CNF1, we eluted RhoA treated with CNF1 *in vitro*, untreated RhoA, or a mixture of both from gels and digested the eluate with trypsin. Separation of the tryptic peptides on a DEAE C-18 HPLC column yielded three major peptides (A,B,C); peptide B in CNF1-treated RhoA eluted slightly later than peptide B from untreated RhoA (Fig. 3a, top and middle panels). In the sample containing both CNF1-treated and untreated RhoA, peptide B is duplicated (Fig. 3a, lower panel). Peptide B1 elutes as peptide B from the untreated RhoA sample, whereas peptide B2 elutes as peptide B from CNF1-treated RhoA (Fig. 3a). Amino-acid sequencing of peptide B from untreated RhoA yielded the sequence QVELALWDTAGQEDYDR, corresponding to amino acids 52–68 of RhoA⁷, whereas in CNF1-treated RhoA the sequence was QVELALWDTAGEEDYDR, which differs only by having a glutamic acid residue at position 63 instead of glutamine. Accordingly, in the CNF1-treated and untreated RhoA mixture, the amino-acid sequence of the B1 peptide corresponded exactly to RhoA 52–68, whereas the B2 peptide had the single Q63E modification of CNF1-treated RhoA. Apart from Gln 63, RhoA contains two additional glutamine residues in positions 29 and 52 (ref. 7) which were

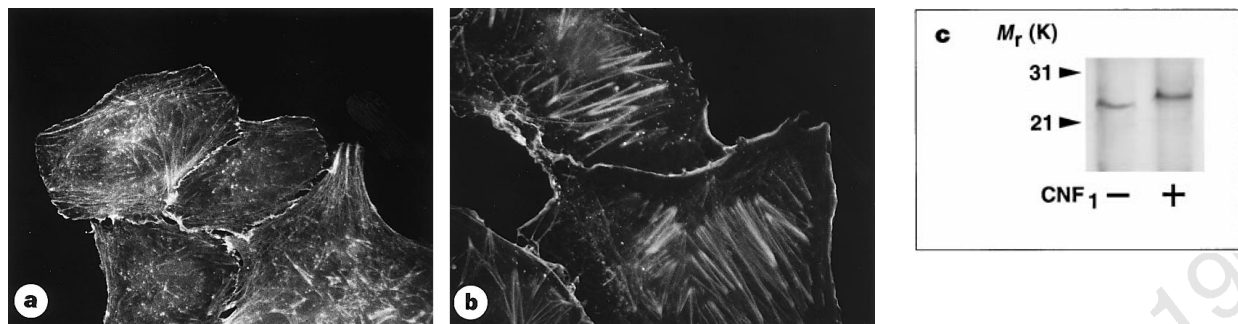


Figure 1 Induction of actin stress fibres in Vero cells by CNF1 and modification of the electrophoretic mobility of the Rho GTP-binding protein by CNF1 *in vivo*. **a**, **b**, Vero cells were treated with 10^{-10} M CNF1 for 18 h, fixed with 4% paraformal-

hyde, permeabilized with 0.05% Triton X-100 and then stained for F-actin with FITC-phalloidin. **a**, Control cells; **b**, cells incubated with CNF1. **c**, Mobility-shift of Rho in control and CNF1-treated Vero cells.

Figure 2 CNF1-induced mobility-shifting of RhoA *in vitro*. RhoA was incubated with CNF1 and processed for electrophoresis. As a control, RhoA or CNF1 was denatured by treatment at 95°C for 15 min, then incubated with either native CNF1 or RhoA.

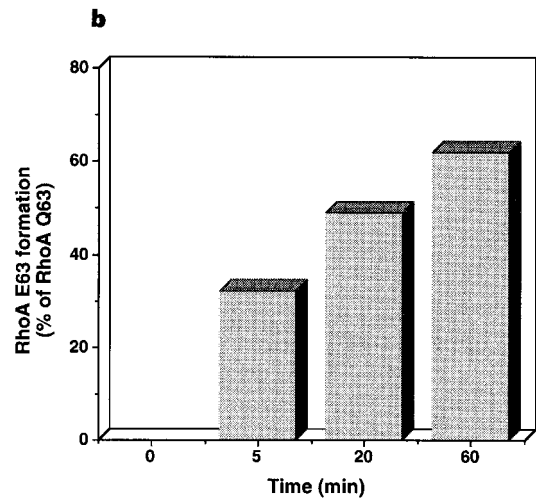
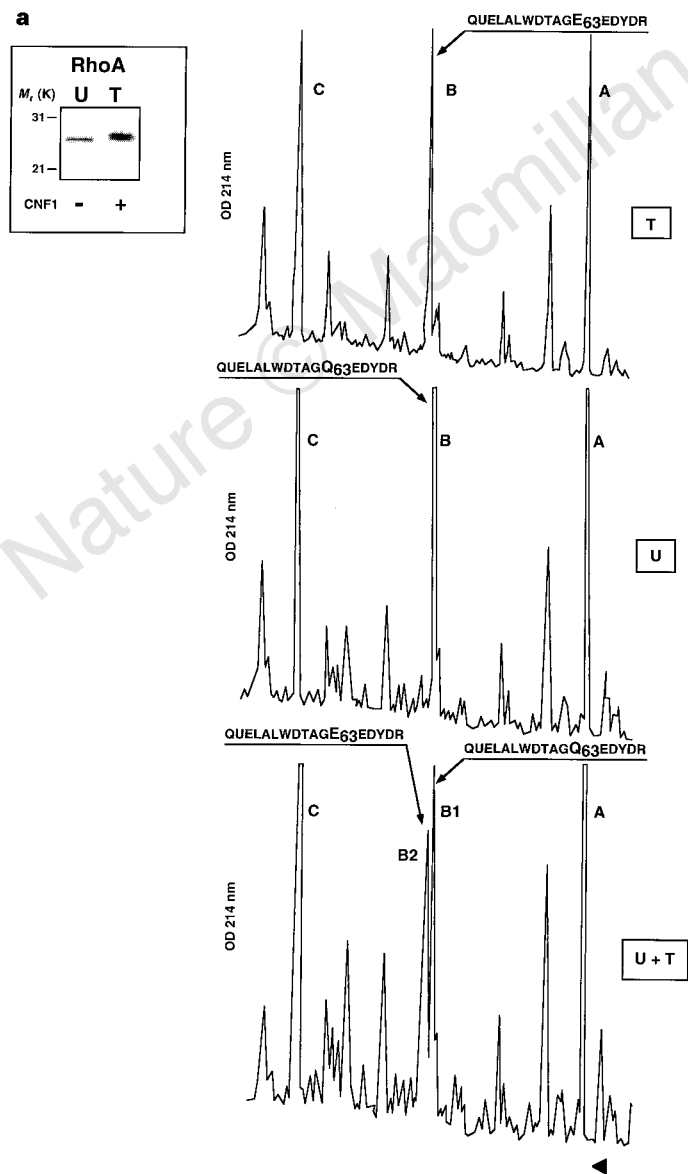
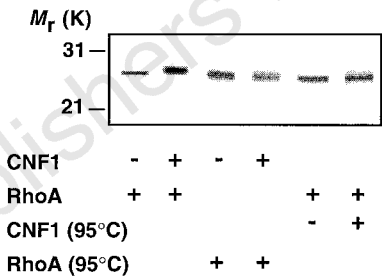


Figure 3 Deamidation of Rho glutamine 63 by CNF1. **a**, Peptide chromatograms of RhoA incubated with (T) or without (U) CNF1, and of a mixture of both (U + T). The amino-acid sequence of the RhoA tryptic peptide spanning amino acids 52–68 is indicated by arrows. Arrowhead indicates the direction of peptides elution. **b**, Time course of CNF1 deamidase effect on RhoA, as studied by formation of peptide 52–68 of RhoA with glutamic acid at position 63.

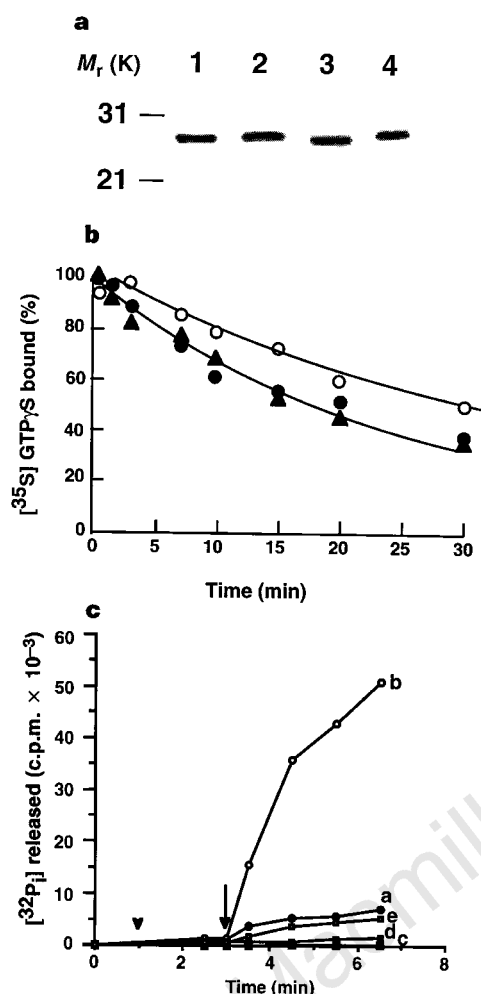


Figure 4 Electrophoretic mobility shift, time course of $[^{35}\text{S}]\text{GTP-}\gamma\text{S}$ dissociation, and determination of the intrinsic and rhoGAP-stimulated GTP hydrolysis of RhoA wild type, Q63E RhoA, and CNF1-treated RhoA. **a**, Electrophoretic mobilities of: 1, RhoA; 2, CNF1-treated RhoA; 3, RhoA; 4, Q63E RhoA. **b**, Dissociation of $[^{35}\text{S}]\text{GTP-}\gamma\text{S}$ in 1 mM MgCl_2 from RhoA (white circles) Q63E RhoA (black circles) and CNF1-treated RhoA (triangles). **c**, Time course of intrinsic and rhoGAP-stimulated GTP hydrolysis. Reactions are described in Methods. Equal loading of the different GTP-binding proteins with $[^{32}\text{P}]\text{GTP}$ was verified by filtration, as described for the dissociation assay. MgCl_2 was added 1 min after the onset of the reaction to initiate GTP hydrolysis (arrowhead). GST-rhoGAP was added 3 min after the onset of the reaction (arrow); final concentration, 10 nM. Line a, RhoA minus rhoGAP; line b, RhoA plus rhoGAP; line c, Q63E RhoA plus or minus rhoGAP; line d, CNF1-treated RhoA minus rhoGAP; line e, CNF1-treated RhoA plus rhoGAP. Results are given in c.p.m. of free ^{32}P . Times are shown in minutes after the start of the reaction.

not modified by treatment with CNF1. Thus, CNF1 acts as a deamidase that specifically modifies Gln 63 of Rho to glutamic acid.

CNF1 must work enzymatically on Rho because the mobility shift induced by CNF1 is blocked by heat denaturation of Rho or of CNF1 (Fig. 2), and because the amount of Q63E RhoA formed depends on the incubation time of Rho with CNF1 (Fig. 3b). Rho deamidation *in vitro* is relatively slow, with only 60% of Q63 RhoA being converted after 1 h (Fig. 3b). It is known that cells need to be incubated with CNF1 for a relatively long time before actin stress fibres accumulate and cell spreading is seen^{3,4,6}. The kinetics of deamidation by CNF1 of Rho *in vitro* were not significantly affected by including cytosolic factors or altering our reaction. Mutations at position 61 of Ras (corresponding to Rho position 63) also modify its apparent electrophoretic mobility^{8,9}.

We next studied the effects of CNF1 on the rate of nucleotide

dissociation, the rate of intrinsic GTP hydrolysis, and the rate of GTP hydrolysis stimulated by the Rho GTPase-activating protein (rhoGAP)¹⁰, and compared them with those of a Q63E RhoA mutant. The electrophoretic mobility of Q63E RhoA was identical to CNF1-modified RhoA (Fig. 4a). Dissociation of $\text{GTP-}\gamma\text{S}$ at 1 mM Mg^{2+} from both CNF1-treated RhoA and mutated Q63E RhoA was slightly increased compared to RhoA (Fig. 4b), with the $\text{GTP-}\gamma\text{S}$ 'off' rate being $k = 0.04 \text{ min}^{-1}$ for both CNF1-modified RhoA and Q63E RhoA, and 0.023 min^{-1} for unmodified RhoA. rhoGAP-stimulated GTP hydrolysis was abolished on Q63E RhoA compared with unmodified RhoA (Fig. 4c). A small residual activity could be detected on CNF1-treated RhoA (Fig. 4c), probably due to the presence of trace amounts of unmodified RhoA. The intrinsic GTPase activity was also lost in the mutant (Fig. 4c). CNF1-modified RhoA and the Q63E mutant have comparable nucleotide affinities and both lack GTPase activity, indicating that they could be identical.

To show that CNF1-treated RhoA and Q63E RhoA behave as activated forms of RhoA, we microinjected both into Vero cells. As wild-type RhoA can induce stress fibres at concentrations close to those required by the permanently active form of RhoA, V14 RhoA¹¹, we determined the minimum concentrations of RhoA, CNF1-modified RhoA and Q63E RhoA needed to induce formation of actin stress fibres as a result of microinjection into Vero cells. Microinjection of Vero cells with RhoA at $50 \mu\text{g ml}^{-1}$ induced maximal stress fibre formation (Fig. 5a), but at $10 \mu\text{g ml}^{-1}$ there was no effect (Fig. 5b). CNF1-modified RhoA (Fig. 5c, d) or Q63E RhoA (Fig. 5e, f) had maximal effect at $25 \mu\text{g ml}^{-1}$ and at $10 \mu\text{g ml}^{-1}$ these effects were still evident (Fig. 5d, f). Thus, CNF1-modified RhoA and Q63E RhoA are both equally effective at inducing the formation of stress fibres. Our results show that CNF1 activates Rho by deamidating Gln 63 to glutamic acid, thereby impairing the intrinsic and rhoGAP-stimulated GTPase. CNF1-modified Rho behaves as the other constitutively active forms of Rho that are mutated at residues 14 or 63.

The Rho family proteins play a key role in actin remodelling in response to growth factors¹². In Swiss 3T3 cells Rho controls actin reorganization into stress fibres and the formation of focal contacts¹³, Rac regulates membrane ruffling¹⁴, and Cdc42 is responsible for the formation of filopodia¹⁵. We have previously shown that CNF1 induces a phagocytic behaviour through activation of membrane ruffling in HEP-2 cells¹⁶. We have now shown that in Vero cells CNF1 induces actin stress fibres but not ruffling (Fig. 1) and decreases the electrophoretic mobility of Rho but not of Rac or Cdc42 (although we cannot rule out the possibility that the PAGE system might not resolve the CNF1-modified forms of Rac and Cdc42). Membrane ruffling may be induced by Rho (in addition to Rac) only in some cell types such as HEP-2 or KB cells when they are stimulated by hepatocyte growth factor or phorbol esters¹⁷. We and others have shown that the invasive bacterium *Shigella flexneri* enters HeLa cells as a result of membrane ruffling triggered by Rho^{18,19}. The dermonecrotic toxin from *Bordetella bronchiseptica* (DNT)²⁰, which shares amino-acid sequence homology with CNF1 (ref. 21), also promotes actin reorganization in cultured cells²². Furthermore, DNT induces a mobility shift of Rho²², suggesting that it also deamidates Gln 63 of Rho. Enzymatic deamidation is a previously undescribed activity of bacterial toxins acting inside host cells; others include ADP-ribosylation, depurination, adenylate cycling, metal-dependent proteolysis and glycosylation. We have now demonstrated that the deamidase activity of CNF1 is responsible for the cytopathic effects of this toxin, and it is likely that this modification is used by other microbial or eukaryotic cell factors to modify regulatory proteins in host cells. □

Methods

Rho electrophoretic mobility-shift assay. Highly purified CNF1¹⁶ was used. Recombinant RhoA was made from the pGEX 2T GST-F25N RhoA plasmid²³ (gift from A. Hall). The GST-RhoA fusion protein was cleaved by thrombin

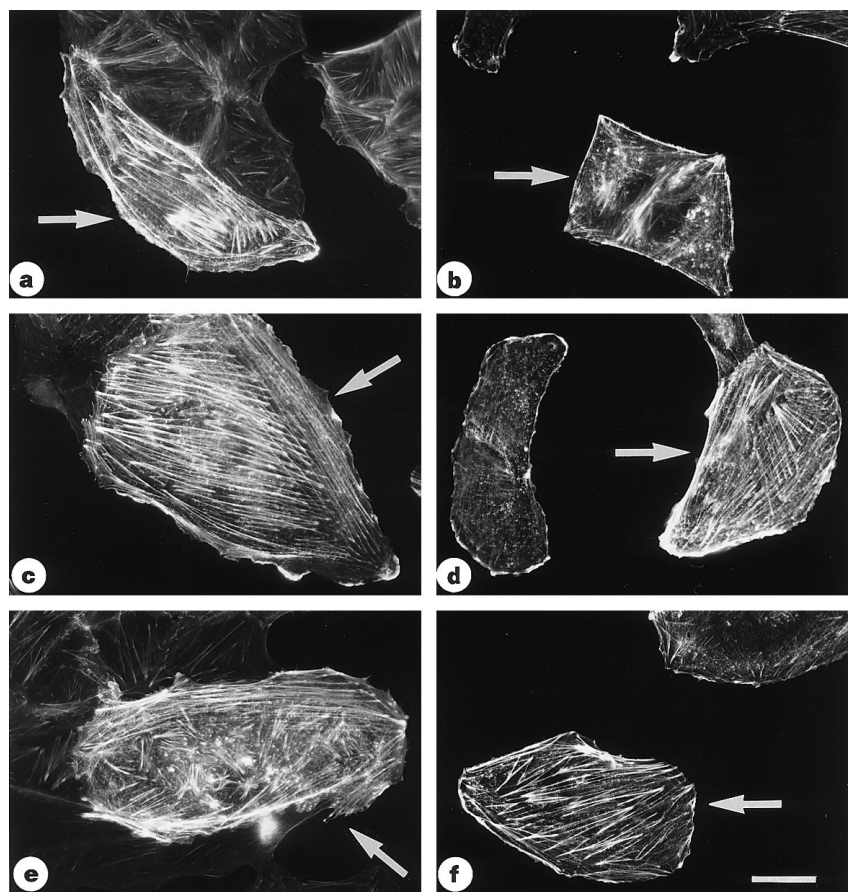


Figure 5 Microinjection into Vero cells of CNF1-treated RhoA, Q63E RhoA and RhoA. **a, b**, Cells microinjected with RhoA (50 $\mu\text{g ml}^{-1}$ in **a**; 10 $\mu\text{g ml}^{-1}$ in **b**). **c, d**, Cells microinjected with CNF1-treated RhoA (**c**, 25 $\mu\text{g ml}^{-1}$; **d**, 10 $\mu\text{g ml}^{-1}$). **e, f**, Cells microinjected with Q63E RhoA (**e**, 25 $\mu\text{g ml}^{-1}$; **f**, 10 $\mu\text{g ml}^{-1}$). Arrows, microinjected cells. Scale bar, 10 μm .

and incubated for 2 h at 37 °C (0.5 μg CNF1 for 2 μg RhoA) in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM MgCl_2 , 10 mM dithiothreitol (DTT) and 100 μM GDP. Samples were boiled for 1 min, electrophoresed on 12% SDS-polyacrylamide gels and stained with Coomassie blue.

Trypsin digestion of gel-eluted Rho, peptide separation and amino-acid sequencing. RhoA was incubated for 2 h at 37 °C with CNF1 (0.5 μg CNF1 for 2 μg RhoA) in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM MgCl_2 , 10 mM DTT and 100 μM GDP, and electrophoresed on a 12% SDS-polyacrylamide gel which was stained with amido-black. Bands corresponding to the Rho protein were cut out of the gel, eluted and digested with 1 μg trypsin in 200 μl 100 mM Tris-HCl buffer (pH 8.8) containing 0.01% Tween 20, for 18 h at 35 °C. Tryptic peptides were separated by HPLC on a DEAE-C18 column using an acetonitrile/trifluoroacetic acid gradient. Peptides were sequenced using an Applied Biosystems microsequencer.

Preparation of the Q63E RhoA mutant. The Q63E mutation was introduced by polymerase chain reaction (PCR) and the plasmid checked by sequencing. Q63E RhoA was expressed in *E. coli* as a GST fusion protein which was then cleaved by thrombin.

GTP- γ S dissociation assay. Q63 RhoA, CNF1-treated RhoA and Q63E RhoA (1 μM final) were loaded with 25 μM [^{35}S]GTP- γ S in a low-magnesium buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 1 mM MgCl_2 , and 2 mM EDTA) at 37 °C for 1 min. The final concentration of free Mg^{2+} was adjusted to 1 mM and 1 mM GTP- γ S was added to initiate [^{35}S]GTP- γ S dissociation. Samples of 25 μl were filtered at the indicated times and radioactivity counted²⁴.

GTPase assay. Q63 RhoA, CNF1-treated RhoA and Q63E RhoA (1 μM final) were loaded with 25 μM [γ - ^{32}P]GTP as described for [^{35}S]GTP- γ S. MgCl_2 (1 mM free Mg^{2+}) was added to start the intrinsic GTPase. For measurements of rhoGAP-stimulated GTP hydrolysis, 10 nM of a 30KC-terminal fragment of GST-rhoGAP (gift from A. Hall²⁵) was added 2 min after MgCl_2 . At the indicated times, aliquots of 25 μl were removed and the $^{32}\text{P}_i$ release was measured by the charcoal method²⁶.

Cell microinjection, immunofluorescence and ADP-ribosylation of Rho. F25N RhoA²³ was used as it is threefold more potent, upon microinjection, in

inducing stress fibres than F25 RhoA¹⁵. CNF1 (12 μg) was incubated with 50 μg recombinant GST-RhoA bound to glutathione beads (Sigma) for 1 h at 37 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM MgCl_2 , 50 mM NaCl, 10 mM DTT, 150 mM KCl (buffer A) supplemented with 100 μM GDP (final volume, 1 ml). The mixture was then packed into a mini-column which was washed with 30 ml buffer A. GST-RhoA was eluted from the glutathione beads with buffer A containing 20 mM reduced glutathione, in 100 μl (containing 0.5 mg ml^{-1} GST-RhoA) and tested for CNF1-induced mobility shift by SDS-PAGE. GST-RhoA (100 μl) was cleaved with 1 μl thrombin (1 U μl^{-1}) (Sigma) for 1 h at room temperature then dialysed against 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM MgCl_2 , 150 mM KCl, 0.1 mM DTT (microinjection buffer (MB)) to remove glutathione. GST was then separated from Rho by binding to glutathione beads and incubating with 10 μl *p*-aminobenzamidine beads (Sigma) to remove thrombin. Before microinjection, the concentration of active GTP-binding proteins present in the RhoA, RhoA Q63E and CNF1-modified RhoA preparations was determined by binding to [^{35}S]GTP- γ S as described²⁴. Preparations of the different GTPases were found to be 60% active. RhoA or Q63E RhoA was diluted with protein A-purified rabbit non-immune antibodies (0.5 mg ml^{-1}) (to determine the efficiency of microinjection) in MB (final volume 20 μl) and microinjected (Transjector 5246 system, Eppendorf) into serum-starved (0.2% FCS, overnight) Vero cells. Microinjections were done in DMEM, which contained 25 mM HEPES buffer to maintain the pH at 7.3. Cells were then incubated at 37 °C for 20 min, fixed with 4% paraformaldehyde, processed for F-actin immunofluorescence with FITC-phalloidin together with rhodamine-labelled anti-rabbit antibodies (Amersham) and examined and photographed with a fluorescence-equipped photomicroscope.

Semi-confluent Vero cells (2×10^6) were incubated or not with 10^{-10} M CNF1 for 18 h then lysed, and lysates were radioactively ADP-ribosylated with *C. botulinum* exoenzyme C₃ as described²⁷.

Received 14 March; accepted 17 April 1997.

1. Caprioli, A., Falbo, V., Roda, L. G., Ruggeri, F. M. & Zona, C. Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological alterations. *Infect. Immun.* **39**, 1300–1306 (1983).

2. De Ryck, J. *et al.* Evidence for two types of cytotoxic necrotizing factor in human and animal clinical isolates. *J. Clin. Microbiol.* **28**, 694–699 (1990).
3. Fiorentini, C. *et al.* Cytoskeletal changes induced in HEp-2 cells by the cytotoxic necrotizing factor of *Escherichia coli*. *Toxicon* **26**, 1047–1056 (1988).
4. Oswald, E. *et al.* Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proc. Natl Acad. Sci. USA* **91**, 3814–3818 (1994).
5. Fiorentini, C. *et al.* *Escherichia coli* cytotoxic necrotizing factor 1 increases actin assembly via the p21 Rho protein. *P. Zbl. Bakt. (suppl.)* **24**, 404–405 (1994).
6. Fiorentini, C. *et al.* *Escherichia coli* cytotoxic necrotizing factor 1: evidence for induction of actin assembly by constitutive activation of the p21 Rho GTPase. *Infect. Immun.* **63**, 3936–3944 (1995).
7. Yeramian, P., Chardin, P., Madaule, P. & Tavittian, A. Nucleotide sequence of human Rho cDNA clone 12. *Nucleic Acids Res.* **15**, 1869 (1987).
8. Der, C. J., Finkel, T. & Cooper, G. M. Biological and biochemical properties of human *ras* H genes mutated at codon 61. *Cell* **44**, 167–176 (1986).
9. Srivastava, S. K., Yuasa, Y., Reynolds, S. H. & Aaronson, S. A. Effects of two major activating lesions on the structure and conformation of human *Ras* oncogene products. *Proc. Natl Acad. Sci. USA* **82**, 38–42 (1985).
10. Garrett, M. D., Major, G. N., Totty, N. & Hall, A. Purification and N-terminal sequence of the p21 Rho GTPase activating protein RhoGAP. *Biochem. J.* **276**, 833–836 (1991).
11. Ridley, A. J. Microinjection of Rho and Rac into quiescent Swiss 3T3 cells. *Methods Enzymol.* **256**, 313–320 (1995).
12. Machesky, L. M. & Hall, A. Rho: a connection between membrane receptor signalling and the cytoskeleton. *Trends Cell Biol.* **6**, 304–310 (1996).
13. Ridley, A. J. & Hall, A. The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389–400 (1992).
14. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. & Hall, A. The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401–410 (1992).
15. Nobes, C. D. & Hall, A. Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular complexes associated with actin stress fibers, lamellipodia and filopodia. *Cell* **81**, 53–62 (1995).
16. Falzano, L. *et al.* Induction of phagocytic behaviour in human epithelial cells by *Escherichia coli* cytotoxic necrotizing factor type 1. *Mol. Microbiol.* **9**, 1247–1254 (1993).
17. Nishiyama, T. *et al.* Rac p21 is involved in insulin-induced membrane ruffling and Rho p21 is involved in hepatocyte growth factor- and 12-O-tetradecanoylphorbol-13 acetate (TPA)-induced membrane ruffling in KB cells. *Mol. Cell. Biol.* **14**, 2447–2456 (1994).
18. Adam, T., Giry, M., Boquet, P. & Sansonetti, P. Rho-dependent membrane folding causes *Shigella* entry into cells. *EMBO J.* **15**, 3315–3321 (1996).
19. Watarai, M., Kamata, Y., Kozaki, S. & Sasakawa, C. Rho, a small GTP-binding protein, is essential for *Shigella* invasion of epithelial cells. *J. Exp. Med.* **185**, 281–292 (1997).
20. Walker, K. E. & Weiss, A. Characterization of the dermonecrotic toxin in members of the genus *Bordetella*. *Infect. Immun.* **62**, 3817–3828 (1994).
21. Falbo, V., Pace, T., Picci, L., Pizzi, E. & Caprioli, A. Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli*. *Infect. Immun.* **61**, 4909–4914 (1993).
22. Horiguchi, Y., Senda, T., Sugimoto, N., Katahira, J. & Matsuda, M. *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesion points by modifying the small GTP-binding protein Rho. *J. Cell Sci.* **108**, 3243–3251 (1995).
23. Self, A. J. & Hall, A. Purification of recombinant Rho/Rac/G25K from *Escherichia coli*. *Methods Enzymol.* **256**, 3–10 (1995).
24. Franco, M., Chardin, P., Chabre, M. & Paris, S. Myristoylation of ADP-ribosylation factor 1 facilitates nucleotide exchange at physiological Mg^{2+} levels. *J. Biol. Chem.* **271**, 1573–1578 (1996).
25. Self, A. J. & Hall, A. Measurement of intrinsic nucleotide exchange and GTP hydrolysis rates. *Methods Enzymol.* **256**, 67–76 (1995).
26. Higashijima, T., Ferguson, K. M., Smigel, M. D. & Gilman, A. G. The effect of GTP and Mg^{2+} on the GTPase activity and the fluorescent properties of G_{α} . *J. Biol. Chem.* **262**, 757–761 (1987).
27. Chardin, P. *et al.* The mammalian G-protein RhoC is ADP-ribosylated by *Clostridium botulinum* C₃ and affects actin microfilaments in Vero cells. *EMBO J.* **8**, 1087–1092 (1989).

Acknowledgements. We thank J. d'Alayer and M. Davi (Institut Pasteur, Paris, France) for trypsin digestion and amino-acid sequencing of Rho proteins; A. Hall for the gift of Rho and RhoGAP expression vectors; J. R. Murphy, E. Van Obberghen-Schilling, P. Cossart and A. Galmiche for discussion.

Correspondence and requests for materials should be addressed to P.B. (e-mail: boquet@unice.fr).

A signature motif in transcriptional co-activators mediates binding to nuclear receptors

David M. Heery, Eric Kalkhoven, Susan Hoare & Malcolm G. Parker

Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

The binding of lipophilic hormones, retinoids and vitamins to members of the nuclear-receptor superfamily modifies the DNA-binding and transcriptional properties of these receptors, resulting in the activation or repression of target genes^{1,2}. Ligand binding induces conformational changes in nuclear receptors and promotes their association with a diverse group of nuclear proteins, including SRC-1/p160 (refs 3–5), TIF-2/GRIP-1 (refs 6, 7)

and CBP/p300 (refs 4, 5, 8, 9) which function as co-activators of transcription, and RIP-140 (ref. 10), TIF-1 (ref. 11) and TRIP-1/SUG-1 (refs 12, 13) whose functions are unclear. Here we report that a short sequence motif LXXLL (where L is leucine and X is any amino acid) present in RIP-140, SRC-1 and CBP is necessary and sufficient to mediate the binding of these proteins to liganded nuclear receptors. We show that the ability of SRC-1 to bind the oestrogen receptor and enhance its transcriptional activity is dependent upon the integrity of the LXXLL motifs and on key hydrophobic residues in a conserved helix (helix 12) of the oestrogen receptor that are required for its ligand-induced activation function¹⁴. We propose that the LXXLL motif is a signature sequence that facilitates the interaction of different proteins with nuclear receptors, and is thus a defining feature of a new family of nuclear proteins.

We have previously shown that the nuclear protein RIP-140 (*M_r* 140,000) interacts with nuclear receptors through at least two distinct sites located at the N and C termini of the protein¹⁵. To map these interaction sites, we examined fragments of RIP-140 fused with a heterologous DNA-binding domain (DBD) for interaction with nuclear receptors in a yeast two-hybrid system. By comparing the sequences of the shortest interacting fragments, we identified a short motif (LXXLL) that was present in all interacting fragments. In total, nine copies of the motif were identified in the RIP-140 sequence, but it was absent in fragments that had no binding activity. To determine whether these short sequences were sufficient to bind to nuclear receptors, we constructed a series of proteins consisting of a DBD fused to 8–10 amino acids which incorporated one copy of each of the nine LXXLL motifs. As shown in Fig. 1a, each of the nine motifs present in RIP-140 made a strong ligand-dependent interaction with the ligand-binding domain (LBD) of the oestrogen receptor (ER), whereas the DBD alone failed to bind. Results were comparable with the LBD of the retinoic acid receptor (RAR) (data not shown). Mutation of hydrophobic residues in the conserved helix H12, which abolishes its ligand-induced activation function (AF-2) and the recruitment of co-activators^{6,10,11,13}, prevented the ligand-dependent interaction of all nine LXXLL motifs with ER (Fig. 1a). We conclude that a short conserved motif contained within as few as eight amino acids is sufficient to bind to transcriptionally active nuclear receptors.

Secondary structure analysis using the Phd program¹⁶ predicts that each motif in RIP-140 is α -helical, with the conserved leucines forming a hydrophobic face. To determine the sequence constraints on functional interaction, we carried out a partial mutational analysis of one motif (amino acids 935–943; Fig. 1b). Western blot analysis indicated that there was no significant variation in the expression of the fusion proteins (data not shown). Mutation of valine at residue 935 to alanine gave ~10-fold reduction in reporter activity in the presence of ligand, which, as the first amino acid is hydrophobic in seven of the nine LXXLL motifs in RIP140, may indicate a preference for the hydrophobic residue at that position. Mutation of any one of the three conserved leucine residues L936, L939 or L940 to alanine resulted in a complete loss of binding to the LBD of ER (Fig. 1b) and RAR (data not shown), emphasizing their importance in mediating the interaction with nuclear receptors. In contrast, mutation to alanine of L941 (which is not conserved among the motifs; Fig. 2a), had no effect on the ability of this sequence to bind to the ER LBD. Replacement of a conserved leucine residue with a valine was tolerated at L936, but not at L939 or L940, indicating that hydrophobic character alone is not sufficient to maintain interaction with ER (Fig. 1b). Amino acids K937, Q938, S942 and E943 were not subjected to mutagenesis as they are not conserved among the motifs we have identified (Fig. 2a).

The steroid receptor co-activator SRC-1 is a truncated protein which interacts with the progesterone receptor through a 196-amino-acid C-terminal region³. We found that the eight most C-terminal amino acids fit the LXXLL consensus, and indeed this

sequence (DBD-SRC1a 1,434–1,442) showed strong ligand-induced binding to ER, but not to the ER H12 mutant (Fig. 1a). Full-length SRC-1 (SRC-1a) proteins from mouse (1,459 amino acids)^{4,5} and human (1,441 amino acids; E.K. *et al.*, manuscript in preparation) an additional nuclear-receptor interaction region between residues 569 and 789 (ref. 5) and 570 and 780, respectively (E.K. *et al.*, manuscript in preparation). Three copies of the LXXLL motif were identified in this central interaction domain of human SRC-1a (Fig. 2a), each of which bound in a ligand-dependent manner to both ER (Fig. 1a) and RAR (data not shown), but not the ER H12 mutant. Interestingly, the sequences and relative positions of the three motifs in the central domain of SRC-1a are conserved in the related co-activator protein TIF-2 (transcriptional intermediary factor 2) (Fig. 2a,b), and correspond to the region of TIF-2 that binds to nuclear receptors⁶. However, unlike SRC-1a, TIF-2 lacks a motif at its C terminus. In addition, we noted that SRC-1 contains three other sequences matching the LXXLL consensus. The motif at residues 45–53, within the basic helix–loop–helix domain at the N terminus of SRC-1a⁵, is predicted by the Phd program to be α -helical, but interacts only very weakly (sixfold) with liganded ER (Fig. 1a) or RAR (not shown), which is consistent with the lack of strong nuclear-receptor-binding activity (E.K. *et al.*, manuscript in preparation). The sequences at residues 111–118 and 913–920, which contain prolines and are unlikely to adopt α -helical structure, did not interact with liganded ER (Fig. 1a), indicating that appropriate secondary structure is probably a prerequisite for binding of LXXLL sequences to nuclear receptors.

CBP/p300 proteins, originally identified as co-activators for the transcription factor CREB^{17,18}, are co-activators for many other transcription factors, including nuclear receptors^{4,5,8,9}, and may integrate several signalling pathways⁴. CBP binds directly to nuclear receptors through its N-terminal 101 amino acids⁴, and has a possible retinoid-receptor (RXR)-specific binding site located between residues 356 and 495 (ref. 8). Our analysis shows that the CBP sequence harbours copies of the LXXLL motif at positions 68–75 and 356–364, which are conserved in the p300 sequence (amino acids 80–90 and 341–351; Fig. 2a). When tested in the two-hybrid assay, the N terminus of CBP (amino acids 1–101; data not shown) and the LXXLL motif at residues 68–75 of the CBP sequence (Fig. 1a) show ligand-dependent binding to ER, but not to the transcriptionally defective ER mutant (Fig. 1a).

To show that the binding of co-activator proteins to nuclear receptors depends on LXXLL motifs, we introduced alanine substitutions in SRC-1a at the conserved leucine couplets at residues 636/7, 693/4, 752/3 and 1438/9 to generate a mutant protein (SRC1a-M1234) in which all four functional binding motifs were disabled. We then compared the ability of *in vitro* translated SRC-1a and SRC1a-M1234 to bind to the LBD of the mouse ER fused to glutathione S-transferase (GST–AF2). Whereas wild-type SRC-1a protein showed ligand-dependent binding to GST–AF2, SRC1a-M1234 did not bind (Fig. 3a). To confirm that the structure of SRC1a-M1234 was not disrupted by the mutations, we compared the interaction of the *in vitro* translated proteins with amino acids 2,058–2,163 of CBP, defined as the SRC-1 binding domain⁴. Both proteins still bound strongly to GST–CBP (Fig. 3a), indicating that this function was maintained in the mutant SRC-1 protein. In addition, increasing concentrations of a peptide (P1) corresponding to the motif at the C terminus of SRC-1a competed with SRC-1a for binding to GST–AF2, but a mutant peptide (P2) did not (Fig. 3b).

To show that LXXLL motifs are necessary for the function of SRC-1 *in vivo*, we compared the abilities of wild-type and mutant SRC-1 to enhance the activity of mouse ER in transient transfection experiments. Unlike SRC-1, which enhanced the activity of the ER, the SRC-1 mutant reduced the transcription of the reporter gene (Fig. 3c). This apparent dominant-negative property of the mutant is probably due to its ability to interact with CBP but not with nuclear

receptors (Fig. 3a). Given that SRC-1 and CBP/p300 may exist as a complex *in vivo*⁹ and that CBP can also bind nuclear receptors^{4,8}, our results suggest that interaction between nuclear receptors and CBP does not compensate for the inability of the SRC-1a mutant protein to bind nuclear receptors, at least under conditions.

The sequences of other nuclear-receptor-binding proteins contain one or more copies of the LXXLL motif (Fig. 2a). The incidence of this motif in the sequences of RIP-140, SRC-1a, TIF-1, TIF-2, CBP and p300, together with the boundaries of the known receptor interaction domains in these proteins, is shown in Fig. 2b. The motif was also identified in several other proteins that may interact with nuclear receptors, including Ara70 (ref. 19), SW13 (ref. 20) and the RelA (p65) subunit of NF- κ B²¹, although the receptor-interaction

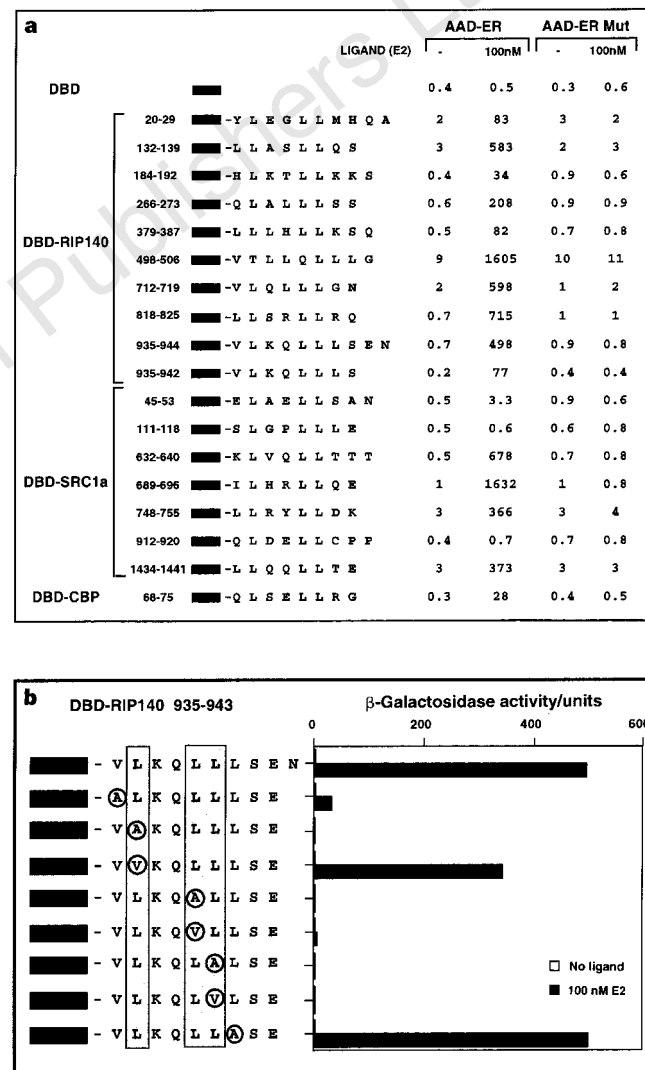


Figure 1 Interaction of LXXLL motifs derived from co-activators with the ER. **a**, The ability of LXXLL motifs, derived from the proteins RIP-140, SRC-1a and CBP, to interact with the LBDs of wild-type or mutant ER was determined by two-hybrid analysis in yeast. DBD-LXXLL proteins were coexpressed with AAD-ER or AAD-ER Mut, which consist of an acidic activation domain (AAD) fused to the LBD of wild-type ER, or a transcriptionally defective ER mutant, respectively. Reporter activities were determined in the presence or absence of 10^{-7} M 17- β -oestradiol (E2) and expressed as units of β -galactosidase activity. **b**, Effects of mutations in the RIP-140 LXXLL motif located at amino acids 935–943 on binding of AAD-ER. Conserved leucine residues are boxed and mutated residues are circled. The reporter activity was determined in the presence (black bars) or absence (white bars) of 10^{-7} M E2.

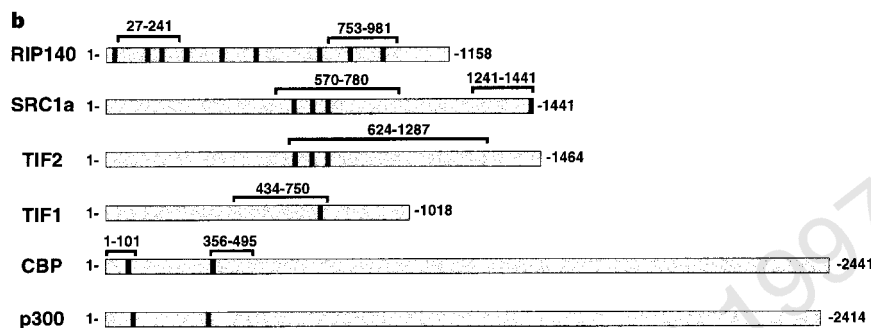
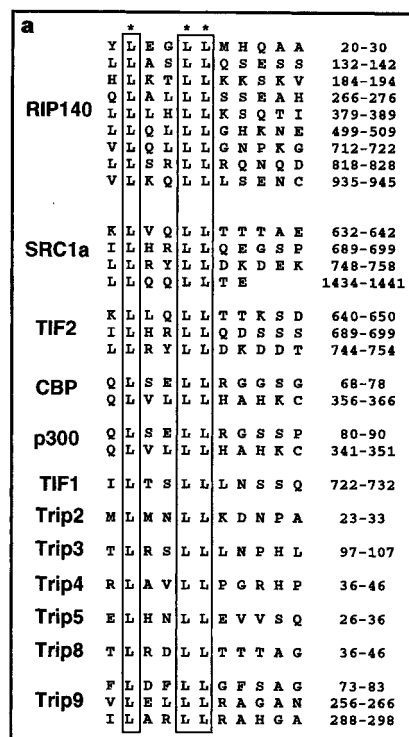


Figure 2 The LXXLL sequence is a signature motif in proteins that bind the LBDs of nuclear receptors. **a**, Alignment of LXXLL motif sequences present in human RIP-140 (ref. 10), human SRC-1 (our unpublished data), mouse TIF-2 (ref. 6), mouse CBP¹⁷⁸ p300 (ref. 26), mouse TIF-1 (ref. 11) and human TRIP proteins¹². The conserved leucines are boxed and the amino-acid numbers are given for each motif. **b**, The incidence of LXXLL motifs (black bars) in sequences of proteins that bind nuclear receptors. The amino-acid boundaries of the nuclear-receptor binding sites are also shown.

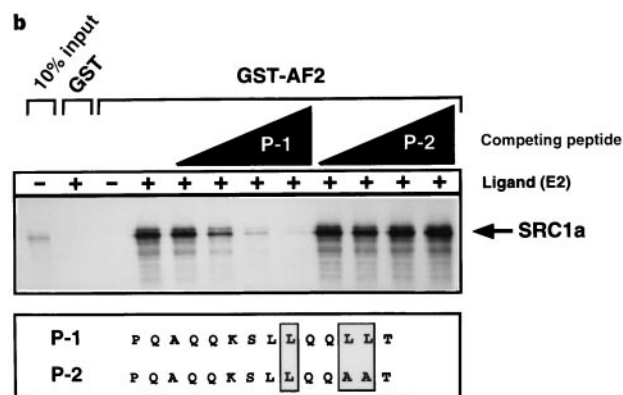
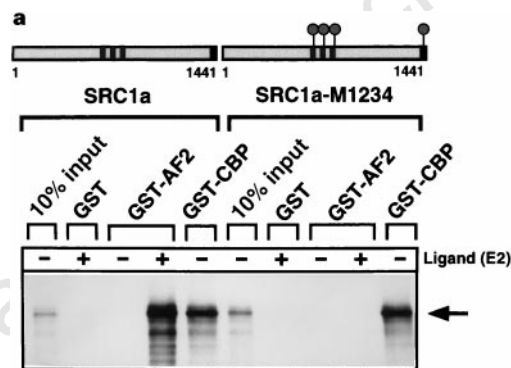
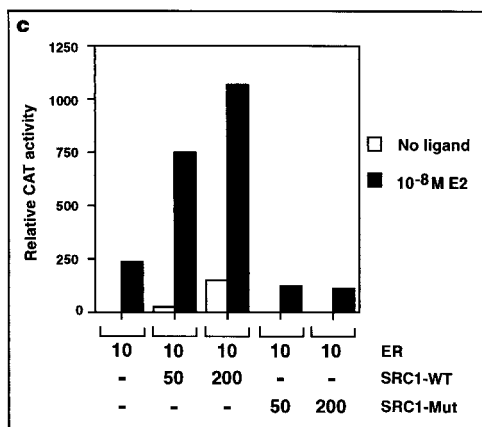


Figure 3 LXXLL motifs are required for binding of SRC-1 to the ER LBD *in vitro* and for the ability of SRC-1 to enhance ER activity *in vivo*. **a**, Wild-type (SRC-1a) and mutant (SRC1a-M1234) SRC1 proteins. Black bars represent the approximate locations of the LXXLL binding motifs in the linear SRC-1a sequence; black circles indicate sites of mutation of LXXLL binding motifs in which conserved leucine residues are replaced by alanines. Binding of wild-type SRC-1a or SRC1a-M1234 mutant to GST alone, to the LBD (amino acids 313-599) of ER (GST-AF2), or the SRC-1 binding domain (amino acids 2,058-2,163) of CBP (GST-CBP) in the presence (+) or absence (-) of 10^{-6} M E2. Signals obtained with 10% of the input of ³⁵S-labelled wild-type and mutant SRC-1 proteins are shown. **b**, The ability of increasing amounts of peptides P1 and P2 to compete with wild-type SRC-1a for binding to GST-AF2 in the presence of ligand. Sequences of the P1 and P2 peptides are given underneath; conserved leucines and alanine substitutions are boxed. **c**, Wild-type but not mutant SRC-1 potentiates activation by ER of the reporter gene 2ERE-pS2-CAT in transiently transfected HeLa cells. Reporter activities obtained from extracts of transfected cells grown in the absence or presence of ligand (10^{-8} M E2). The amounts of ER, SRC1-WT and SRC1-Mut expression plasmids used for transfection are indicated (ng DNA). Activities were averaged from duplicates.



domains in these proteins have not been mapped. The ability of other proteins containing LXXLL motifs to bind to nuclear receptors will depend on their subcellular localization, as well as on the α -helicity and surface-accessibility of the motifs.

We have identified a signature sequence that is present in a group of diverse nuclear proteins, including co-activators, and which is necessary and sufficient for their binding to liganded nuclear receptors. We have shown that the motif is essential for the function of SRC-1 as a coactivator of ER *in vivo*. As many nuclear-receptor-binding proteins contain multiple copies of the LXXLL motif, it remains unclear whether this facilitates the contact of partners in homo- and heterodimeric nuclear receptors, or whether it provides an alternative interaction surface to accommodate conformational changes imposed by the binding of nuclear receptors to different response elements. Systematic mutation of LXXLL motifs in co-activators such as SRC-1 and CBP should reveal the crosstalk or synergy that exists between different signal transduction pathways and provide a better understanding of their proposed roles as co-activators and integrators.

Note added in proof: A recent study has shown that residues 722–732 of the protein TIF-1 α mediate its binding to nuclear receptors (ref. 27). □

Methods

Two-hybrid interaction assays. The yeast reporter strain used for all two-hybrid assays was W303-1B (HML α MAT α HMRA his3-11,15 trp-1 ade2-1 can1-100 leu2-3,11, ura3), carrying the plasmid pRL Δ 21-U3ERE which contains a *lacZ* reporter gene driven by three oestrogen-response elements (EREs)²². Plasmids pBL1 and pASV3, which express the human ER DBD and the VP16 acidic activation domain (AAD), respectively²³, were used to generate DBD or AAD fusion proteins for two-hybrid interaction analysis. DBD–LXXLL motif fusion proteins were generated by ligation of phosphorylated annealed oligonucleotide pairs into the pBL1 vector. AD–ER was constructed by cloning a PCR fragment encoding amino acids 282–595 of the human ER into pASV3. AAD–ER Mut was constructed similarly, except that amino acids M543 and L544 or ER were mutated to alanines by recombinant PCR. All fusion constructs were fully sequenced. Transformants containing the plasmids were obtained by selection for the appropriate plasmid markers and were grown to late log phase in 15 ml selective medium (yeast nitrogen base, containing 1% glucose and appropriate supplements) in the presence or presence of 10^{-7} M 17- β -oestradiol (E2). Expression of DBD and AAD fusion proteins in yeast cell-free extracts was verified by immunodetection using a monoclonal antibody recognizing the human ER (gift from P. Chambon). The antibody recognizes the 'F' region of the LBD in the human ER, and also the 'F' region tag at the N termini of the DBD fusion proteins²³. Equal amounts of protein were electrophoresed on polyacrylamide gels and transferred to nitrocellulose for western blotting. Preparation of cell-free extracts by the glass-bead method and β -galactosidase assay of the extracts were done as described²². Two-hybrid experiments were repeated several times, and results shown in Fig. 1a,b are reporter activities measured in a single representative experiment. β -Galactosidase activity is expressed as nmol min⁻¹ per μ g protein.

Assay of *in vitro* binding and peptide inhibition. GST–AF2 consists of the LBD of the mouse ER (amino acids 313–599) fused to GST²⁴. GST–CBP consists of GST fused to the SRC1-binding domain of CBP and was constructed by cloning a PCR fragment encoding residues 2,058–2,163 of mouse CBP into the vector pGEX2TK (Pharmacia). Human SRC-1 α and SRC-1 ϵ cDNAs were isolated from a human B-cell cDNA library and cloned into a modified version of the expression vector pSG5. SRC-1 α M1234 were constructed by recombinant PCR to introduce the leucine-to-alanine mutations at positions 636/637, 693/694, 752/753, and 1,438/1,439 or 636/637, 693/694 and 752/753, respectively. ³⁵S-labelled SRC-1 proteins were generated by *in vitro* translation and tested for interaction with GST proteins in the presence or absence of 10^{-6} M oestradiol (E2) as described²⁵. Peptide P1 or P2 at 4 mg ml⁻¹ was added to GST-binding reactions immediately before the ligand. The increasing

concentrations of peptide added in competition experiments were 2.5, 5, 12.5 and 25 μ M.

Transient reporter assays. HeLa cells were transfected with 1 μ g reporter 2ERE–pS2-CAT²⁵, 150 ng β -galactosidase expression plasmid (internal control), 10 ng ER expression plasmid, and 50 or 200 ng SRC1 expression plasmids or empty vector per well (in duplicate) using 24-well plates. Transfected cells were incubated overnight in DMEM medium without phenol red and containing 10% charcoal-treated FBS, and washed in fresh medium before addition of ligand (10^{-8} M E2) or vehicle. After 40 h, cells were collected and extracts analysed for chloramphenicol acetyltransferase (CAT) and β -galactosidase activities^{14,15}. β -Galactosidase activity was used to correct for differences in transfection efficiency.

Received 10 December 1996; accepted 8 May 1997.

1. Beato, M., Herrlich, P. & Shutz, G. Steroid-hormone receptors—many actors in search of a plot. *Cell* **83**, 851–857 (1995).
2. Mangelsdorf, D. J. *et al.* The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839 (1995).
3. Onate, S. A., Tsai, S. Y., Tsai, M.-J. & O'Malley, B. W. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1357 (1995).
4. Kamei, Y. *et al.* A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403–414 (1996).
5. Yao, T.-P., Ku, G., Zhou, N., Scully, R. & Livingston, D. M. The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. *Proc. Natl Acad. Sci. USA* **93**, 10626–10631 (1996).
6. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P. & Gronemeyer, H. TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.* **15**, 101–108 (1996).
7. Hong, H., Kohli, K., Trivedi, A., Johnson, D. L. & Stallcup, M. R. Grip1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone-binding domains of steroid-receptors. *Proc. Natl Acad. Sci. USA* **93**, 4948–4952 (1996).
8. Chakravarti, D. *et al.* Role of CBP/p300 in nuclear receptor signalling. *Nature* **383**, 99–103 (1996).
9. Hanstein, B. *et al.* p300 is a component of an estrogen receptor coactivator complex. *Proc. Natl Acad. Sci. USA* **93**, 11540–11545 (1996).
10. Cavaillès, V. *et al.* Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J.* **14**, 3741–3751 (1995).
11. Le Douarin, B. *et al.* The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptor, is fused to B-raf in the oncogenic protein T18. *EMBO J.* **14**, 2020–2033 (1995).
12. Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A. & Moore, D. D. Interaction of thyroid-hormone receptor with a conserved transcription mediator. *Nature* **374**, 91–94 (1995).
13. vom Baur, E. *et al.* Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. *EMBO J.* **15**, 110–124 (1996).
14. Danielian, P. S., White, R., Lees, L. A. & Parker, M. G. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J.* **11**, 1025–1033 (1992).
15. L'Horsset, F., Dauvois, S., Heery, D. M., Cavaillès, V. & Parker, M. G. RIP-140 interacts with multiple nuclear receptors by means of two distinct sites. *Mol. Cell. Biol.* **16**, 6029–6036 (1996).
16. Rost, B. & Sander, C. Improved prediction of protein secondary structure by use of sequence profiles and neural network. *Proc. Natl Acad. Sci. USA* **90**, 7558–7562 (1993).
17. Kwok, R. P. S. *et al.* Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370**, 223–226 (1994).
18. Arias, J. *et al.* Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**, 226–229 (1994).
19. Yeh, S. & Chang, C. Cloning and characterization of a specific coactivator, ARA₇₀, for the androgen receptor in human prostate cells. *Proc. Natl Acad. Sci. USA* **93**, 5517–5521 (1996).
20. Yoshinaga, S. K., Peterson, C. L., Herskowitz, I. & Yamamoto, K. R. Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors. *Science* **258**, 1598–1604 (1992).
21. Stein, B. & Yang, M. X. Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF- κ and C/EBP β . *Mol. Cell. Biol.* **15**, 4971–4979 (1995).
22. Metzger, D., Losson, R., Bornert, J.-M., Lemoine, Y. & Chambon, P. Promoter specificity of the two transcriptional activation functions of the human oestrogen receptor in yeast. *Nucleic Acids Res.* **20**, 2813–2817 (1992).
23. Le Douarin, B., Pierrat, B., vom Baur, E., Chambon, P. & Losson, R. A new version of the two-hybrid assay for detection of protein–protein interactions. *Nucleic Acids Res.* **23**, 876–878 (1995).
24. Cavaillès, V., Dauvois, S., Danielian, P. S. & Parker, M. G. Interaction of proteins with transcriptionally active estrogen receptors. *Proc. Natl Acad. Sci. USA* **91**, 10009–10013 (1994).
25. Montano, M. M., Ekena, K., Krueger, K. D., Keller, A. L. & Katzenellenbogen, B. S. Human estrogen-receptor ligand activity inversion mutants—receptors that interpret antiestrogens as estrogens and estrogens as antiestrogens and discriminate among different antiestrogens. *Mol. Endocrinol.* **10**, 230–242 (1996).
26. Eckner, R. *et al.* Molecular cloning and functional analysis of the adenovirus E1A associated 300 kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.* **8**, 869–884 (1994).
27. Le Douarin, B. *et al.* A possible involvement of TIF1 α and TIF1 β in the epigenetic control of transcription by nuclear receptors. *EMBO J.* **15**, 6701–6715 (1996).

Acknowledgements. We thank our colleagues for discussion and for communicating unpublished results; P. Freemont, N. Jones and A. Parker for their suggestions and for critically reading the manuscript; P. Chambon for pBL1, pASV3 and the anti-ER monoclonal antibody; B. Katzenellenbogen for 2EREppS2-CAT; N. Jones for the yeast strain; K. Hobbs for oligonucleotides; N. O'Reilly for peptides; W. Bessant for photography; and G. Clark for automated sequencing. D.M.H. and E.K. were supported by grants from the European Community TMR program and the Netherlands Organisation of Scientific Research (NWO), respectively.

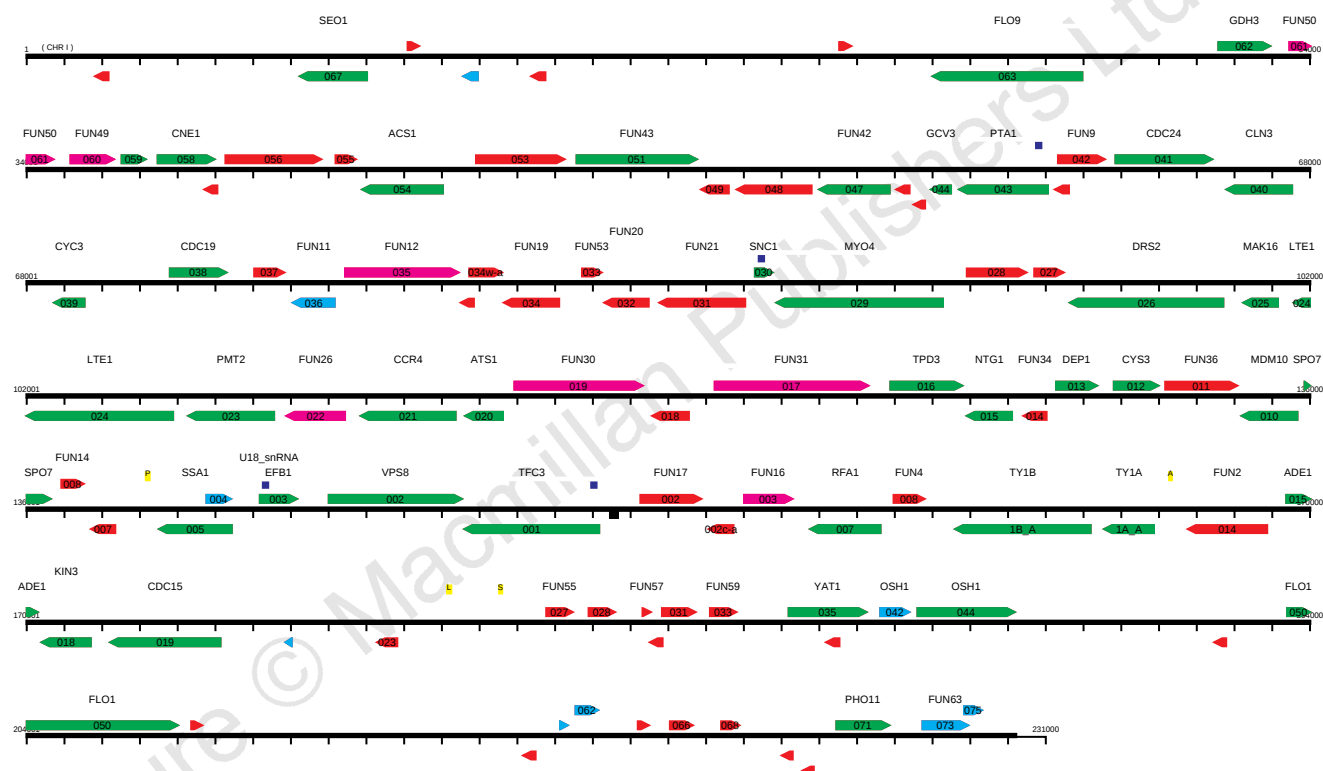
Correspondence and requests for materials should be addressed to M.G.P. (e-mail: m.parker@icrf.icnet.uk).

Overview of the yeast genome

H. W. Mewes, K. Albermann, M. Bähr, D. Frishman, A. Gleissner, J. Hani, K. Heumann, K. Kleine, A. Maierl, S. G. Oliver, F. Pfeiffer & A. Zollner

Nature 387 (Suppl.) 9 (1997)

The display for chromosome 1 was incorrect as published owing to an error in the production process. The correct figure is shown here. □



Microorganisms under scrutiny

A microcystin ELISA, two blood culture systems, a confocal instrumentation reference, an automated agar sterilization and plate pouring system, and anaerobic workstations are among the products covered in this feature.

Cytosensor microphysiometer

From Molecular Devices

The 'Work Reference Guide' is the title of a 20-page brochure containing some 150 scientific references describing the uses of the microphysiometer

These applications include receptor activation and inactivation, signal transduction elucidation, agonist profiling, experimental *in vitro* toxicology, microbial metabolism, and growth factor, neurotrophic factor and cytokine responses. The reference guide also lists over 100 different cell types evaluated in the system, which is designed to detect responses triggered by a wide variety of receptor classes coupled to different second messengers. These receptor classes include G-protein linked receptors, ligand-gated ion channels and tyrosine kinase receptors activated via growth factors, cytokines and neurotrophic factors.

Reader Enquiry No. 100

Basic Z

From Constant Systems

A continuous cell disrupter for bacterial, fungal, mammalian, plant or algal cells

With flow rates from 30 ml to 80 ml min⁻¹, the disrupter provides general disruption and release of subcellular particles, as well as the release of intracellular particles such as nucleic acids and proteins. The unit features a push-button start, an easy-to-clean design with no 'consumable' parts, a cooling facility, selective breakage, scale-up capability and no aerosol production. A 'one-shot' head adaptor is included for use with non-flowing samples and quantities from 1 ml to 10 ml. Disruption is achieved through the use of high pressure, forcing



The Basic Z disrupter from Constant Systems.

the sample through a small fixed orifice at high speed, without an increase in temperature. The pressure is adjustable up to 2.7×10^5 kPa and is stated to be consistent and stable during the disruption cycle.

Reader Enquiry No. 101

Confocal instrumentation reference

From Bio-Rad

A new 152-page bibliography cites a further 600 references that include the use of the company's confocal instrumentation

Research fields that have shown particularly strong recent growth include bacteriology/microbiology, pathology, embryology, neurobiology and biochemistry, especially drug discovery, says Bio-Rad. The reference includes full contact information for authors, as well as details of the fluorochromes and probes used in each paper. Available free of charge, the bibliography may prove to be a valuable reference source for research applying confocal microscopy. Other subject areas covered include genetics, materials science, experimental microscopy, parasitology and plant biology.

Reader Enquiry No. 102

Microcystin ELISA kit

From Wako

According to the manufacturer, the kit offers a 50 to 1,600 pg ml⁻¹ range of measurement, a sensitivity stated to be approximately 10³ times greater than with HPLC

Results are said to be obtained in as little as four hours, accounting for the presence of virtually all toxic microcystins. Microcystins, a group of cyclic heptapeptide hepatotoxins, are the most commonly reported toxins produced by the bloom-forming cyanobacteria and are a primary cause of cyanobacterial poisoning. Besides their acute toxicity, it was recently reported that microcystin LR has tumour promoting activity in rats, as well as inhibiting protein phosphatase 1 and 2A. From these findings, microcystins have recently called attention to their role as possible environmental tumour promoters. This ELISA is said to offer convenient, solid-phase extraction-free quantification. In the normal assay, formate incubation time is stated to be 18–26 h with a sensitivity of 60 pg ml⁻¹, whereas, the short assay format takes as little as 3 h with a sensitivity of 100 pg ml⁻¹. The double-assay method accommodates 43 samples and the single-assay method 91 samples.

Reader Enquiry No. 103



Sartorius' microbiological monitor comes with presterilized membrane, Petri dish and media.

Microbiological monitor

From Sartorius

A 100-ml microbiological monitor that is designed to lower costs

Sterile and disposable, the monitor has a 47-mm diameter cellulose nitrate membrane with a large bottom port that gives faster flow rates and decreased testing times. It has been designed for the easy detection of microorganisms in beverages and water, but is suitable for many other applications. Available as a complete presterilized package (membrane, Petri dish and media), it is said to do away with time-intensive steps involved in setting up steel funnels, flame sterilizing and preparing media. Another feature of the new unit is the integral magnifying disc on the cover, which enables the user to examine any colonies that have appeared on the membrane surface by simply rotating the disc. The standard monitor package contains 50 sterile disposable monitor units, 50 coloured bottom port plugs and a large funnel adapter and a small funnel adapter.

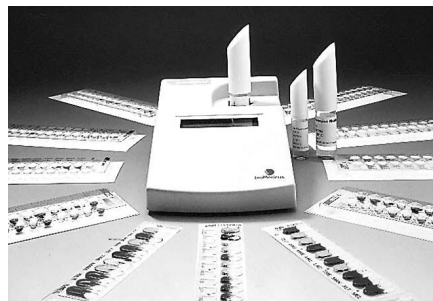
Reader Enquiry No. 104

Densimat

From bioMérieux

A system designed for density of suspensions destined for use with manual or automated identification and susceptibility test strips

The use of this new densitometer should help improve the utility and reliability of



Densimat from bioMérieux is for use with identification and susceptibility test strips.

API, ID32, rapid ID32, ATB, and rapid ATB strips. Easy-to-handle and compact, the Densimat weighs only 0.5 kg and runs on its own integral lithium batteries. Operation is said to be simple — as soon as an ampoule is inserted, the instrument reads the microbial density. The results are then displayed on the LCD panel within the range of 0.5–7.5 McFarland units. A new 44-page, full-colour catalogue describing the entire bioMérieux range of products for industrial and environmental microbiology is now available.

Reader Enquiry No. 105

Media and preparation

Sterile Blood

From TCS Microbiology

Sterile animal blood in PVC bags for cost-effective bacteriological media preparation

With the needs of the larger scale blood user in mind, the company has designed a system that offers a reduction in the contamination risk associated with the use of bottles. The company states that it provides packed-cell volumes with minimal risk, quality-control sampling and reduced wastage. Litre volumes and upward are available and are said to carry the reassurance of adherence to a BS EN ISO 9002 accredited quality assurance system incorporating rigorous visual and chemical QC procedures.

Reader Enquiry No. 106

BBL product brochure

From Becton Dickinson Microbiology Systems

The company offers support in the standardization of prepared media and consumer cost maintenance

The support offered includes helping the clinical laboratory achieve standardization of optimal media, for example, adopting the fewest number of ideal products necessary to achieve the high-quality testing results. A full-colour brochure is available to help the laboratory manager choose from the many plated media formulations that are available.

Reader Enquiry No. 107

Rediprep

From Mast Diagnostics

An extensive range of high-quality prepared media in plate, bottle and tube formats

All media are prepared under BS EN ISO9002 registered conditions and are said to be compliant with laboratory accreditation needs. The company offers microbiology laboratories a flexible service for the supply of poured plates and bottled media. Flexible ordering systems are in place to deal with changing needs, or standing orders can be put into place to ensure continuity of supply. The range includes standard media for general use and specialized media. Other media not listed can be manufactured to order, but are subject to a minimum order requirement. Although specifically designed for UK laboratories, the company will discuss export options with interested customers. The range includes Ready Poured Plates: single media, Bi-plates and contact plates, as well as specialized plates, irradiated media (plates and bottles) and bottled-prepared media.

Reader Enquiry No. 108

AS-10 automatic media preparation

From Brunswick Scientific

An automated agar sterilization and plate pouring system

A quarter turn in and a quarter turn out allows for speedy removal and insertion of the dispense valve and thermowell, as well as a larger, more convenient addition port. Headplate components are smaller so that they are more accessible and easier to handle and the headplate itself is light and easily rotated to give unobstructed access, according to the manufacturer. Media preparation times are said to be faster as well, producing 2–10 l of ready-to-pour, high-quality agar within just one hour. The AS10's stirring mechanism helps to prevent caramelization to ensure high-quality agar. It couples directly with automatic pourer/stackers, inkjet batch identification systems and programmable dispensing pumps to provide a complete, automated service to the busy media preparation laboratory.

Reader Enquiry No. 109

Signal and Isolator

From Oxoid

Two blood culture systems that provide complementary diagnostic approaches for general purpose and specialist applications

Signal is a one-bottle system containing a specially formulated broth medium that allows the culture of aerobes, anaerobes and microaerophilic organisms for general bacteraemia and fungaemia screening. Isolator is a more specialized detection and

isolation system based on direct draw tubes, and is said to be suited for use in cases where fungaemia, low-level bacteraemia or infection by fastidious microorganisms is suspected. In the Signal blood culture system, blood is inoculated into individual bottles, each of which carries a growth indicator device. Gas production from growth activity generates positive pressure in the bottle, displacing some of the blood/broth mixture into the device. Samples can be removed directly from the device for subculturing purposes, without having to enter the bottle itself. According to the manufacturer, the clear detection of microbial growth provided by Signal facilitates the rapid isolation, identification and antimicrobial susceptibility testing of microorganisms from blood culture. Isolator is said to be a rapid and highly sensitive tube-based blood culture system that detects and isolates microorganisms in a single process. Tubes are available in two sizes: an adult version for use with 10-ml blood samples, and a paediatric version that can be used to culture samples ranging from 0.5 ml to 1.5 ml in volume. In both cases, tubes are used for phlebotomy, as well as subsequent sample processing. The manufacturer states that the use of lysis centrifugation and direct plating technology means that colonies are available an average 12–14 h sooner than would be possible with conventional broth-based or biphasic methods.

Reader Enquiry No. 110

Wall Guide

From Sigma

This free wall guide highlights microbial media and components

This easy-to-use, alphabetical reference includes descriptions and other pertinent information on over 85 different microbial media and associated products used in biotechnology. The convenient wall reference lists over three-dozen general and specialized microbial media products, such as brain-heart fusion agar, Luria broth base, and thioglycollate medium. Also featured is a diverse selection of media components, including bacteriological agars, protein hydrolysates and yeast extracts, as well as components for specialized applications.

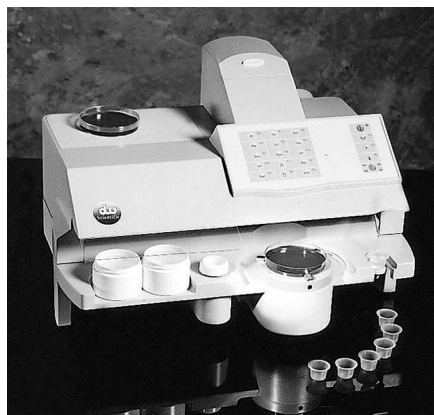
Reader Enquiry No. 111

WASP plater

From Don Whitley Scientific

This automatic spiral plater (WASP) has minimal set up and the option to select and 'lock' user-defined parameters — variable or uniform deposition, fill mode and plate size

A single keystroke initiates the complete cycle, including loading of sample, plating out and cleaning. The microprocessor-



The WASP automatic spiral plater from Don Whitley has a microprocessor-controlled syringe.

controlled sample syringe has a capacity of 250 μ l, which allows flexibility of total volume dispensed and a consequent increase in the machine's sensitivity range. Microprocessor control has the added benefits that multiple plates can be inoculated without the need to refill the syringe. A choice of uniform or variable deposition modes is available for all possible sample volumes. WASP will inoculate a plate at the fastest speed possible for any particular volume, carefully optimizing the centrifugal force to ensure that sample is not pushed to the edge of the plate. After inoculation is complete, the cleaning cycle is automatically initiated, drawing upon fresh sterile water and five per cent hypochlorite solution to ensure effective sterilization of the tip. Users can be removing one plate and preparing the next while cleaning is under way. All parameters are continually monitored by the unit's CPU.

Reader Enquiry No. 112

Incubators and cabinets

ShelLab 2323/TC

From Borolabs

Designed to be a low-cost CO₂ incubator, incorporating a warm air-jacket design that provides temperature uniformity usually only found in application-specific models

The new models have had their exterior redesigned to be easier to use — all control parameters, including calibration, can now be set from the front-mounted touch-pad panel. A parallel printer or computer connection port has also been incorporated to allow users to export data from the incubator to any IBM-compatible printer. The port can also be used for remote monitoring of temperature and CO₂ alarm conditions. Borolabs also offers a free CO₂ changeover unit that will automatically start using a new CO₂ cylinder when one is running low. Such features as an integral microprocessor that ensures precise temperature and CO₂ control regardless of how the chamber is loaded have been retained. Humidification is achieved through evaporation, and rela-

tive humidity is displayed with an accuracy of 1.0 per cent. A 25-mm access port accommodates through-wall operation of electrical equipment within the chamber and a tempered glass viewing door allows the scientist to view samples without loss of temperature, CO₂ or humidity. The other main design feature of the incubator is its triple-wall construction. An extra large waterjacket surrounds the incubator chamber and over this a secondary layer of insulation has been added to the side walls. This design offers temperature uniformity throughout the incubator and helps to eliminate the possibility of cold spots within the chamber which may result in condensation — a primary source of contamination.

Reader Enquiry No. 113

BH12R and BH12D safety cabinets

From Labcaire

Two new class II microbiological safety cabinets designed to meet the airflow and structural requirements of BS 5726 (1992)

With high levels of user protection, the clean sheet design also has low noise levels and ergonomic simplicity. Oversized filters and high-torque fans are said to ensure low noise and high performance, while low-mounted, fully electronic touch controls ensure ease of operation for all users. According to the manufacturer, both units provide sample protection by bathing work surfaces with 0.12- μ m filtered air. A one-piece, crevice-free work surface is said to be easy to clean and helps avoid contamination. Electronics and filters are front mounted to simplify servicing. Moreover, there is optimized internal lighting and a generous 1,200-mm stainless steel work surface to provide a comfortable working environment. Large HEPA filters are sealed under negative pressure to eliminate leaks. The recirculatory



Class II microbiology cabinets from Labcaire.

BH12R and ducted BH12D units share the dimensions 1,500 × 1,340 × 780 mm (H × W × D). Optional stands and other accessories, such as ultraviolet lights and formalin vapourizer units, are available to order.

Reader Enquiry No. 114

Concept anaerobic workstations

From Baker Scientific

There are three models within this range, which includes the Concept Plus, Concept 300 and Bugbox workstations

The largest model in the range is Concept Plus, which requires only 1,600 mm of bench space and has been designed to address the needs of the larger laboratory where sample throughput and incubator capacity are paramount. This unit offers appropriate growth conditions for anaerobes, has a large incubated work area, a storage facility for up to 500 plates with an 'economy' purge cycle for small batch transfer. The Concept 300 has a footprint measuring just 900 × 650 × 750 mm and can accommodate up to 400 plates. A novel tube interlock facilitates transfer of up to 11 × 90-mm Petri dishes in 15 s, whereas a single-plate entry system allows individual dishes to be transferred directly to the cabinet. Standard features of these two models include dual-gas operation, bare-hand access, automatic humidity control and nitrogen change-over with microprocessor monitoring and alarm systems. Completing the range is the Bugbox, a small and sophisticated unit, says the company, which includes all the standard features of the larger workstations and is intended for smaller research projects and clinical laboratories. It accommodates up to 180 plates, making the costly use of anaerobic jars redundant, and offers precise temperature and humidity controls. An optional feature of all Concept units is the connection of a data logging system and chart recorder for 24-h monitoring.

Reader Enquiry No. 115

Odds and ends

Surespin 630

From Sorvall

A swinging-bucket rotor that has been designed to be an easy-to-use, top-loading rotor with greater sample security

The rotor should help to eliminate sample-loading errors in ultracentrifuge operations. Its top-access design makes attachment pins clearly visible, allowing easy verification that each bucket is properly seated and thus enhancing sample protection. The rotor has been built for speeds up to 30,000 r.p.m. In addition, it has cavities that guide buckets easily into place, such that they can be loaded and unloaded without having to remove the rotor body from the centrifuge. Furthermore, bayonet-style covers require no special tools to seal. The Surespin rotor



Sorvall's Surespin 630 swinging-bucket rotor.

is designed for compatibility with other competitive ultracentrifuges.

Reader Enquiry No. 116

Centricon Plus-20 and -80

From Millipore

Part of the company's family of centrifugal filtration devices

These devices are easy-to-use and may replace stirred-cell systems, dialysis and other methods that require time to set-up, maintenance procedures or additional processing steps. The devices incorporate the high-flow Biomax membrane for fast processing or the high-recovery YM membrane. They also offer the invert spin method of concentrates recovery, allowing for improved sample recovery. Centricon Plus-20 concentrates samples of 20 ml down to 150 µl in as little as 10 min; whereas, Centricon Plus-80 allows for concentration of 80 ml down to 300 µl in as little as 30 min. These devices fit any 50 ml and 250 ml swinging bucket rotors.

Reader Enquiry No. 117

MasterAmp kit

From Epicentre Technologies

PCR kits and enzymes incorporate a new technology that is designed to improve PCR

According to the manufacturer, amplification consistency and yields are increased, and poor, unreliable or failed amplification reactions are eliminated with the company's kits and enzymes. This includes templates that cannot be amplified using conventional PCR because of GC-rich regions or extensive secondary structure. Other MasterAmp products include PCR optimization kits,



MasterAmp PCR kits from Epicentre.

PCR core kits, and PCR-qualified *Taq*, *Tfl*, *Tth* and AmpliTherm DNA polymerases.

Reader Enquiry No. 118

Ultrapure RapidGel solutions

From Amersham

A wide range of premixed liquid acrylamide to simplify DNA sequencing

These solutions have been formulated to allow DNA sequencing gels to be poured in minutes and still retain high levels of reproducibility. Stated to be suitable for both radioactive and fluorescent sequencing electrophoresis, the solutions are stable, premixed and available in a range of convenient percentages in either TBE (4, 4.75, 5, 6 and 8 per cent) or GTG (4, 6 and 8 per cent) formulations; a 40 per cent stock concentrate is also available. The RapidGel-XL high-resolution matrix is for use in DNA sequencing and is stated to yield up to 40 per cent more readable sequence per gel. It is available either as ready-to-use solutions (4, 6 and 8 per cent) or in a 40 per cent stock concentrate, which can be used for the preparation of custom percentages.

Reader Enquiry No. 119

FP500 Econo

From Ultra-Lum

A new modular gel system for economical, efficient photography, documentation and analysis of gels

This low-cost system uses a high-resolution CCD camera mounted on the UltraLum low-profile darkroom. The darkroom is designed to fit on any UltraLum transilluminators and can be equipped with optional ultraviolet lighting, incident or epi-illumination. Incremental, modular additions can be made with a 256 gray-scale thermal printer, for example, providing photography at less than ten cents a picture, or a frame grabber that will plug into either an IBM- or Macintosh-formatted computer for complete documentation and analysis. The PHD 2000 can be installed providing documentation capability with image enhancement. The K33000 option can convert this basic setup into a full visualization, documentation and analysis system.

Reader Enquiry No. 120

Multichannel Gel Loading Syringes

From Hamilton

Syringes that allow users to transfer up to 12 samples at a time from microwell plates to even the thinnest electrophoresis gels

There are three available needle sizes, including a new 0.2-mm design for deeper loading between plates and into wells. Adjustable volume stops allow faster and more accurate reloading and improve reproducibility. The syringes allow quick



Hamilton's multichannel syringes allow the efficient transfer of 4, 6, 8 and 12 samples.

transfer of 4, 6, 8 and 12 samples at a time from microwell plates to sequencing gels. Adjustable volume stops allow faster, more accurate reloading and excellent reproducibility. No disposable pipette tips need to be changed or oriented to fit. Models for 4- or 8-channel transfer by microwell plate row and 6- or 12-channel transfer plate column are available. Gas-tight syringes are said to ensure accuracy of greater than 99 per cent, and adjustable stops allow for fast, reproducible volume transfers of 0.5–10 µl.

Reader Enquiry No. 121

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

ADVERTISEMENT

CUSTOM HYBRIDOMA DEVELOPMENT

- Ascites Production
- Purification • Conjugation
- Polyclonal Antibodies

Also Available

1-800-481-9737

e-mail: antibodies@htibio.com
<http://www.htibio.com>
 fax: (760) 788-9694
 P.O. Box 1319
 Ramona, CA 92065

HTI Bio-Products, Inc.